

**MOLECULAR AND *IN VITRO* GROWTH COMPARISONS OF
Encephalitozoon hellem ISOLATES FROM HUMAN AND BIRD HOSTS**

A Thesis

by

PAULETTE FRANCESCA WATERS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2003

Major Subject: Veterinary Microbiology

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May 2003

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ABSTRACT

Molecular and *In Vitro* Growth Comparisons of *Encephalitozoon hellem* Isolates from Human and Bird Hosts. (May 2003)

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Molecular and *in vitro* comparisons were performed using two isolates of *Encephalitozoon hellem*, one from an avian host and one from a human host, and one isolate of *Encephalitozoon cuniculi* from a rabbit. The molecular comparisons were performed by amplifying and sequencing the gene coding for a zinc metallo-aminopeptidase from cDNA and gDNA obtained from each of the isolates. The *E. hellem* sequences shared >99 % identity between each other and 70% identity with the *E. cuniculi* sequences. Conserved HEXXH and GXMEN motifs located within the sequences classify the protein as an aminopeptidase of the M1 family, with at least one zinc atom required for catalytic activity.

In vitro growth comparisons of the isolates described above were performed under simulated “mammalian and avian conditions”. The models utilized mammalian and avian cell lines and sera at incubation temperatures of 37 °C and 40 °C, respectively. Three separate experiments were performed. *E. cuniculi* grew best under the mammalian model and significantly better than both *E. hellem* isolates under this model. The *E. hellem* isolates were able to infect and replicate under both the mammalian and avian models, which reflects the zoonotic potential of these isolates.

DEDICATION

This work is dedicated with much love to my family. Thank you Dad, Mom and Luly for your love and support, without which this work would not have been accomplished much less imagined.

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I would like to thank Dr. Holman for her encouragement, friendship, and mentorship. In addition, I would like to extend a special thanks to Dr. Holman for extending her help whenever needed regardless of the hour. I would also like to thank my committee members Dr. Karen Snowden and Dr. Louis Abbott for their guidance and instruction in the completion of this work. Also, thanks to Dr. Kerry Barling for his expertise in performing statistical analyses. I thank Kathleen Logan for her assistance with the microsporidia cultures. I thank Doug Melendy and Justin McCain for excellent technical support.

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CHAPTER I

INTRODUCTION

Microsporidia is a non-taxonomic designation that describes parasites belonging to the phylum Microspora. Over 1000 different species have been identified belonging to approximately 140 genera. Members of nearly every phylum of the animal kingdom may be infected by these obligately intracellular parasites including invertebrates, fish, mammals, birds, and man (reviewed by Weiss, 2001).

Microsporidia are spore forming parasites that range in size from 1 to 12 μm . Spore shape is generally constant for members of the same genus. The most common spore shape is oval, however, other spore shapes include rod-like and bent forms. The spore wall consists of three regions: 1) an outer electron-dense exospore, 2) an electron-lucent endospore consisting of a protein-chitin complex, and 3) an inner membrane that surrounds the sporoplasm. The exospore may be smooth or ridged, with or without filaments, tubular structures, or mucous coating containing filaments. A unique characteristic of microsporidia is the polar filament, also known as the polar tubule. This filament encircles the infective parasitic cytoplasm, or sporoplasm, within the spore. Depending on the species, the filament can form between 4 to 30 coils in single or several rows. Upon activation the polar filament is referred to as the polar tubule, which provides the means for infecting a new host cell (Vavra & Larsson, 1999).

This thesis follows the style and format of *Parasitology*.

The basic life cycle of microsporidia can be divided into three phases: infective, proliferative, and sporogonic. The infective phase includes the spore in the environment until the sporoplasm is injected into the appropriate host cell. Germination of the spore may be induced by chemical or physical changes in the environment. It is these changes that trigger the polar filament to evert into the polar tubule. It is the polar tubule that is responsible for piercing the host cell and transferring the sporoplasm into the host cell. Once the sporoplasm is injected into the appropriate host cell, the proliferative phase of the life cycle begins in which the sporoplasm replicates by merogony producing meronts. Sporogony follows the proliferative phase, in which meronts develop into sporonts. These sporonts replicate by binary or multiple fission into sporoblasts, which in turn develop into mature spores completing the life cycle (Cali & Takvorian, 1999).

Microsporidia are classified as eukaryotic, possessing a nucleus with a nuclear envelope, an intracytoplasmic membrane system, and chromosome separation on mitotic spindles. However, unlike eukaryotes, microsporidia lack mitochondria and centrioles (Weiss, 2001). Microsporidian ribosomes resemble the 70S prokaryotic organelle, consisting of a large 23S subunit and a small 16S subunit (Weiss, 2000). Yet the rRNA genes of microsporidia are shorter than eukaryotic or prokaryotic genes (Vossbrinck *et al.* 1987). Microsporidia also lack the 5.8S ribosomal RNA subunit and ITS2 region typical of eukaryotes. Instead a region homologous to the 5.8S subunit is fused at the beginning of the large ribosomal subunit, which is a typical feature of bacteria (Peyretilade *et al.* 1998).

The genomes of microsporidia are small, ranging from 2.3 to 19.5 Mb (Weiss & Vossbrinck, 1999). The smallest eukaryotic genome yet described is that of the microsporidian *Encephalitozoon intestinalis* (2.3 Mb) (Peyretailade *et al.* 1998). Analysis of the genome of *Encephalitozoon cuniculi* shows a low abundance of repetitive DNA and low gene copy number (Biderre *et al.* 1995). Furthermore, only one intron in a microsporidian gene has been identified. The intron is 28 base pairs long and located at the beginning of the large subunit ribosomal protein of *E. cuniculi* (Biderre, Metenier & Vivares, 1998). This loss of genetic material is most reasonably connected with the obligate parasitism of these organisms (Biderre *et al.* 1995).

The first microsporidian species to be recognized was *Nosema bombycis*, a parasite of silkworms, which devastated the silk industry in the early 19th century. Microsporidia were initially only known to be problematic in invertebrates and fish and not until later known to infect vertebrates and humans (Wittner, 1999). In 1922 Wright and Craighead identified the first mammalian microsporidian infections in laboratory rabbits (Wright & Craighead, 1922). Subsequently, in 1923 a new genus and species of microsporidia named *Encephalitozoon cuniculi* by Levaditi and others was recognized as the cause of encephalitis in laboratory rabbits (Wittner, 1999). *E. cuniculi* has since been identified in numerous different mammals including humans, and other microsporidia species have since been reported in mammals and humans as well (reviewed by Deplazes, Mathis & Weber, 2000).

A wide range of microsporidia has been successfully cultivated *in vitro* including those of invertebrate and vertebrate hosts. Initially attention primarily

focused on microsporidia of economic importance such as *Nosema apis*, a parasite of honey bees, and *Glugea stephani*, a parasite of flounder (Visvesvara, 2002). However, in 1969 the first culture of a mammalian microsporidia was established when Shadduck successfully continually propagated *E. cuniculi* of rabbit origin in rabbit kidney (RK) cells (Shadduck, 1969). Between 1969 and 1990, *E. cuniculi* was the only microsporidia of mammalian origin cultivated *in vitro* (Visvesvara *et al.* 1999). In 1990 cultures of *Vittaforma corneae* from corneal scrapings of a human patient were established (Shadduck *et al.* 1990). In 1991 Didier and others established continuous cultures of three *Encephalitozoon hellem* isolates from AIDS patients suffering from keratoconjunctivitis (Didier *et al.* 1991b).

A number of isolates of *E. cuniculi* (Hollister *et al.* 1995; Deplazes *et al.* 1996; Mathis *et al.* 1997; Rossi *et al.* 1998; del Aguila *et al.* 2001) and *E. hellem* (Didier *et al.* 1991a; Hollister *et al.* 1993; Deplazes *et al.* 1998) have been established in culture. Isolates have been maintained in a variety of different cell types and media. Different cell lines that have been used for the cultivation of *Encephalitozoon* spp. include rabbit kidney cells (RK), Madin-Darby canine kidney (MDCK), monkey kidney (E6), human lung fibroblast (HLF), lung fibroblasts (MRC-5), fetal bovine lung fibroblasts (FBF), and human colon carcinoma (Caco-2). Also a variety of culture media has been used for propagating *Encephalitozoon* spp., including RPMI 1640, Eagle's minimal essential medium (EMEM), and Dulbecco's modified Eagle's medium (DMEM), each supplemented with either 5 % or 10 % fetal bovine serum (FBS) and

antibiotics and incubated at 37 °C. Cultures have been successfully maintained with or without 5 % CO₂ (reviewed by Visvesvara, 2002).

In 1959 the first case of microsporidiosis in a human was documented (Matsubayashi *et al.* 1959). Sporadic cases of microsporidiosis in humans were reported between 1959 and 1985 (Margileth *et al.* 1973; Marcus, Van der Walt & Burger, 1973; Sprague, 1974), however, it was not until the 1980's that microsporidia gained attention as causing disease in man. During the mid and late 1970's increasing numbers of cases of severe diarrhea were reported in male homosexuals. Although the etiologic agents in most of these cases were identified as *Salmonellae*, *Shigellae*, and *Giardiae*, in a portion of these cases with large-volume diarrhea the causative agent was not identified, and the patients did not respond to antibiotic therapy. The syndrome in these cases was sometimes described as wasting disease (Wittner, 1999).

In 1985 several reports described microsporidian parasites in acquired immunodeficiency syndrome (AIDS) patients suffering from chronic diarrhea (Dobbins, & Weinstein, 1985; Modigliani *et al.* 1985; Desportes *et al.* 1985). Also in 1985, *Enterocytozoon beineusi*, a new microsporidian parasite, was identified in AIDS patients (Desportes *et al.* 1985). Consequently, microsporidiosis became recognized as a disease affecting the immunoincompetent. Subsequently, human cases of microsporidiosis caused by *E. cuniculi*, *E. intestinalis* and *E. hellem* were increasingly reported (Wittner, 1999). To date several other genera have been identified in human infections, including *Nosema*, *Vittaforma*, *Brachiola*, *Trachipleistophora*, and *Pleistophora* (Weiss, 2001).

Microsporidia are opportunistic pathogens most often associated with human immunodeficiency virus (HIV) infection and AIDS patients; however, cases have also been documented in immune suppressed individuals and occasionally in immune competent persons (Shadduck *et al.* 1990; Davis *et al.* 1990; Theng *et al.* 2001). The most frequently identified microsporidia in humans are *E. bienersi*, *E. intestinalis*, *E. hellem*, and *E. cuniculi*. *E. bienersi* and *E. intestinalis* can invade intestinal enterocytes resulting in chronic diarrhea in the infected patients. In addition, *E. intestinalis*, as well as *E. hellem* and *E. cuniculi*, have been described in disseminated infections. In human patients, *E. cuniculi* is associated with encephalitis and hepatitis, while *E. hellem* infections are normally associated with keratoconjunctivitis, sinusitis, respiratory disease, and prostatic abscesses (Weiss, 2001).

Members of the genus *Encephalitozoon* are uninucleate and have an approximate spore size that ranges between 1.0 and 2.5 μm , with 4-9 coils of the polar filament arranged in a single row. Spores are oval in shape and the exospore is uneven (Wasson & Peper, 2000). All *Encephalitozoon* spp. replicate inside parasitophorous vacuoles within host cells and have a disporogonic life cycle producing two spores for every sporoblast. *E. intestinalis* secretes a fibrillar network that gives the appearance that the vacuole is septate. This feature can discriminate *E. intestinalis* from *E. cuniculi* and *E. hellem*, which are morphologically indistinguishable (Didier *et al.* 1991b).

The first reports of encephalitozoonoses in man were described as *E. cuniculi* based on the morphological characteristics of the parasite (Wittner, 1999). However, morphology is only sufficient for genus identification, not species differentiation, as was shown by the characterization and subsequent naming of *E. hellem* (Didier *et al.* 1991a). In 1991, a new species was identified by immunological and protein analysis of isolates obtained from three AIDS patients suffering from keratoconjunctivitis. These isolates were distinguishable from *E. cuniculi*, *Nosema corneum* and *Nosema algerae*, but not from each other, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis. As a result Didier and others proposed the name *E. hellem* for the isolates (Didier *et al.* 1991a).

After 1991, the cause of subsequent cases of encephalitozoonosis in human patients was identified as *E. hellem*. Doubt arose as to whether *E. cuniculi* was correctly identified in all cases of encephalitozoonosis described prior to 1991 and if *E. cuniculi* could infect man. But in 1995 *E. cuniculi* infection in man was documented by molecular analysis. However, question still remains as to whether *E. cuniculi* was actually the cause of all cases described prior to 1991, because the identification was based solely on morphology (Franzen & Muller, 1999).

It is speculated that microsporidiosis in humans may be zoonotic. Microsporidia that infect humans have been reported in a number of different animals. *E. bieneusi* has been identified in pigs, cattle, cats, macaques, rabbits, and dogs (Mansfield *et al.* 1997; Breitenmoser *et al.* 1999; Dengjel *et al.* 2001). *E. cuniculi* has been reported in rabbits, rodents, carnivores, and monkeys (Wasson & Peper, 2000).

Mammalian infections of *E. intestinalis* have been identified in dogs, donkeys, cows, goats, and pigs (Bornay-Llinares *et al.* 1998). There is no direct evidence of animal to human transmission, but molecular analyses may serve as a helpful indicator of zoonoses. Molecular markers can be used to analyze human and animal isolates to determine if they are genetically the same, thus implicating the ability to cross infect different hosts.

Microsporidia infections have also been documented in different species of birds. Reports based on morphologic identification of microsporidia in a number of avian hosts refer to the parasites as microsporidia, *Encephalitozoon*-like, or *Encephalitozoon* spp., but in these cases a parasite species was not identified. The first described case identified *Encephalitozoon* in masked lovebirds (*Agapornis personata*) by histopathology and electron microscopy (Kemp & Kluge, 1975). Since then, morphologic identification of the genus *Encephalitozoon* has been reported in the following bird species: peach faced lovebirds (*Agapornis roseicollis*) (Novilla, 1978; Lowenstine & Petrak, 1980; Branstetter & Knipe, 1982; Norton & Prior, 1994), masked lovebirds (*Agapornis personata*) (Randall *et al.* 1986; Powell *et al.* 1989), Fischer's lovebirds (*Agapornis fischeri*) (Randall *et al.* 1986), double yellow-headed Amazon parrots (*Amazona ochrocephala*) (Poonacha, William & Stamper, 1985; Canny *et al.* 1999), and an ostrich (*Struthio camelus*) (Gray, Puette & Latimer, 1998). Clinical infections were described in each of these cases except for the ostrich, which was not a clinical case but slaughtered for routine inspection. In addition, many of the clinical cases involved young birds displaying signs such as anorexia, weakness, and

loose droppings, which presented approximately one month after purchase (Lowenstine *et al.* 1980; Poonacha *et al.* 1985; Randall *et al.* 1986).

Increasingly, molecular tools are being used to supplement morphologic identification of microsporidia. Comparison of the small subunit ribosomal RNA (SSUrRNA) gene has been widely used as a molecular marker for species identification. The SSUrRNA gene is highly conserved among species and is useful for interspecies identification. The internal transcribed spacer region (ITS) of the ribosomal RNA genes is less conserved and, therefore, is more variable between and within species and is a successful marker for strain typing (Adam *et al.* 2000). These tools not only provide a means for species and strain differentiation, but may also be helpful as indicators of zoonoses.

In 1995, Didier and colleagues identified differences in eight *E. cuniculi* isolates from 3 rabbits, 3 mice, and 2 dogs through molecular sequencing of the ITS region (Didier *et al.* 1995). Differences in the ITS region were significant enough to group the isolates into three strains. Sequence analysis revealed a 5'-GTTT-3' sequence that was repeated 2, 3 or 4 times within the ITS sequence, depending on the isolate. The isolates were grouped and typed as follows: strain type I, rabbit isolates, ITS sequence containing three 5'-GTTT-3' repeats; strain type II, mouse isolates, ITS sequence containing 2 repeats; and strain type III, dog isolates, ITS sequence containing 4 repeats.

Further sequence analyses of human and animal *E. cuniculi* isolates indicated strain type I and strain type III may be zoonotic. Strain type I is associated with rabbits

and has been identified from human isolates from Italy and Switzerland. Strain type III has been identified from dogs and humans as well (Deplazes *et al.* 2000; Mathis *et al.* 1997). An isolate of strain type III of domestic dogs was first identified from an AIDS patient in 1996 (Didier *et al.* 1996). More recently, several studies have identified *E. cuniculi* strain type III by ITS sequence analysis from isolates obtained from additional dogs and from human patients worldwide (Snowden, Logan & Didier, 1999; Weitzel *et al.* 2001; del Aguila *et al.* 2001; Tosoni *et al.* 2002).

Similar heterogeneity exists among *E. hellem* isolates as demonstrated by ITS and SSUrRNA nucleotide sequencing (Mathis *et al.* 1999; Xiao *et al.* 2001). Between 1993 and 1998, identical ITS regions were published for 4 *E. hellem* isolates originating from the USA and Germany (Vossbrinck *et al.* 1993; Katiyar *et al.* 1994; Franzen *et al.* 1998). However, in 1999 2 additional genotypes were described by Mathis *et al.* after sequencing the ITS regions of *E. hellem* isolates from Swiss and Tanzanian HIV patients (Mathis *et al.* 1999). The isolates were referenced against the previously known ITS sequences, classified as genotype I. The ITS sequences from 2 of the Swiss patient isolates and the Tanzanian patient isolate were identical, but differed from genotype I by 5 base pair substitutions, 3 single nucleotide deletions, and an extra poly T stretch at the 3' end, and was therefore classified as genotype II. One of the substitutions was a G for an A, resulting in a tetrameric 5'-GTTT-3' in place of 5'-ATTT-3'. The last sequence, also a Swiss isolate, contained this particular base pair substitution but with an additional 5'-GTTT-3' repeat, and was classified as genotype type III. Genotype III was identical to genotype II except for

this additional repeat. In summary, 2 Swiss and 1 Tanzanian isolate were genotype type II, and 1 Swiss isolate was genotype III based on GTTT repeats found within ITS sequences, similar to the genotypes of *E. cuniculi* (Mathis *et al.* 1999).

Partial sequencing of the SSUrRNA gene and/or ITS regions identified *E. hellem* in different bird species. In 1997, molecular analysis using polymerase chain reaction (PCR) and Southern blotting identified *E. hellem* as the species responsible for infection of budgerigars (Black *et al.* 1997). Partial sequencing of the SSUrRNA gene also identified *E. hellem* in two eclectus parrots (Pulparampil *et al.* 1998), an ostrich (Snowden & Logan, 1999), and a clinically normal peach faced lovebird (Snowden, Logan & Phalen, 2000). The ITS region was used as a molecular marker for identification of *E. hellem* in a wild yellow streaked lory, which was the first case to identify *E. hellem* in a wild caught bird (Suter *et al.* 1998). *E. hellem* was also identified in hummingbirds by sequencing portions of the SSUrRNA and the ITS region (Snowden, Daft & Nordhausen, 2001). The sequencing results of these various isolates showed 99% to 100% identity with *E. hellem* human isolate sequences. Analysis of the ITS regions from different bird isolates, including hummingbird (Snowden *et al.* 2001), a peach faced lovebird (Snowden *et al.* 2000), and a yellow streaked lory (Suter *et al.* 1998), characterized them as genotype I.

The gene coding for the polar tubule protein (PTP) has also been sequenced from *E. hellem* isolates as a tool for molecular genotyping. In a 2001 study of 24 isolates, the ITS region and the SSUrRNA and PTP genes were sequenced and compared (Xiao *et al.* 2001). The PTP sequences showed the highest resolution,

grouping the isolates into 4 genotypes. The differences in these sequences were due to a 60 base pair repeat that was repeated 6, 7, 8, or 3 times for genotypes 1A, 1B, 2A, or 2B, respectively. The PTP genotype 2B sequence also included five 66 base pair repeats and an 18 base pair insert upstream of the repeat region (Xiao *et al.* 2001).

Interestingly, in this same study the ITS region had less variability than the SSUrRNA genes sequenced. Based on ITS region and SSUrRNA gene sequence analysis, the isolates had 2 and 3 genotypes, respectively. The isolates were obtained from biopsy, bronchoalveolar (BAL), sputum, and urine samples from 20 human patients worldwide including the USA (11), Puerto Rico (1), Italy (10), Switzerland (1), and Spain (1). Sequence analysis of the ITS region showed that all the isolates, except for the Switzerland urine isolate, were identical to genotype 1 described previously by Mathis *et al.* (1999), and the Switzerland isolate was similar, but not identical, to genotypes 2 and 3 reported by Mathis *et al.* (1999). Sample site, i.e., urine, sputum, etc., had no apparent correlation with the obtained genotypes (Xiao *et al.* 2001). Further molecular and epidemiologic studies are needed to assess the significance of these results, and isolates obtained from birds should be included. Currently ITS genotype 1 is the predominant genotype of human and bird isolates (del Aguila *et al.* 2001; Xiao *et al.* 2001).

Based on an increasing collection of data, it is probable that birds are the natural hosts of *E. hellem*. As noted above, the organisms have been identified in a number of different bird species, including both wild birds (Suter *et al.* 1998) as well as pet birds (Barton, Phalen & Snowden *et al.* 2000). *E. hellem* infections have been

described in both symptomatic and asymptomatic birds. In addition, concurrent bacterial (Powell *et al.* 1989; Black *et al.* 1997) and viral infections (Pulparampil *et al.* 1998) were also documented in clinically ill birds suggesting that microsporidia may not otherwise cause disease in birds, but rather that birds may be asymptomatic carriers. Although the majority of cases in which *E. hellem* was identified in birds involved psittacine birds, the identification of *E. hellem* from an ostrich, gouldian finch and hummingbirds shows that *E. hellem* infections are not restricted to members of the parrot family. The higher detection of *E. hellem* in psittacine birds is most likely attributed to the popularity of psittacine birds as pets, which are more likely to receive veterinary attention when ill.

Molecular comparisons of SSUrRNA and ITS sequences from human and avian isolates show greater than 99 % identity. Thus, it has been suggested that *E. hellem* may be a zoonotic parasite and that birds may be the natural host of *E. hellem* (Black *et al.* 1997; Pulparampil *et al.* 1998; Canny *et al.* 1999; Snowden & Logan, 1999). However, further evidence is needed to validate this hypothesis, so the current study was undertaken using molecular and *in vitro* growth studies to address this question. Previously, *in vitro* growth comparisons have been performed with the mosquito microsporidian parasite *N. algerae* (Trammer *et al.* 1999). The first studies were performed to determine if *N. algerae* could infect mammalian cells and replicate at mammalian temperatures in order to predict the ability of these microsporidia to infect humans (Undeen, 1975). A recent study by Trammer *et al.* (1999) comparing the *in vitro* infectivity and replication of *N. algerae* in mammalian cells at 31 °C and

38 °C showed that it took longer before infected cells could be detected and that a lower number of spores were produced at 38°C. That study indicates that *in vitro* models can be used to show the preferred host conditions of these parasites, because deviations from their natural host conditions can result in delayed replication and a decrease in the number of spores being produced.

In vitro growth comparisons were performed in the current study using 2 different models, 1 simulating mammalian host conditions and 1 simulating avian host conditions, in order to compare the growth of 2 different isolates of *E. hellem*. An *E. hellem* isolate cultured from a bird (Snowden *et al.* 2000) and an isolate cultured from a human infection (Didier *et al.* 1991a) were compared to each other and to an isolate of *E. cuniculi* (rabbit isolate, gift of John Shadduck), a well-characterized mammalian parasite, which served as a control.

Molecular comparisons of the bird and human *E. hellem* isolates and *E. cuniculi* were performed in this study. A gene coding for a metallo-aminopeptidase was chosen as an additional molecular marker to evaluate homology between the 2 *E. hellem* isolates. This gene is of particular interest because previous studies have indicated that inhibition of aminopeptidases may serve as an effective treatment of microsporidiosis. The sequence data obtained from this study will be valuable in future drug design and treatment of microsporidiosis.

The gene coding for a zinc metallo-aminopeptidase was sequenced from bird and human isolates of *E. hellem* and a rabbit isolate of *E. cuniculi*. The obtained sequences were aligned and compared for percent homology. Furthermore, the

obtained *E. cuniculi* aminopeptidase sequence was also compared to the sequence from a different isolate of *E. cuniculi* in the GenBank database (GenBank accession no. CAA06646). Inter- and intragenic variation was evaluated among the isolates.

CHAPTER II

MOLECULAR COMPARISONS

Introduction

Microsporidia are spore forming intracellular parasites found worldwide with a wide host range (Mathis, 2000). These parasites are opportunistic pathogens known to cause disease in immunocompromised individuals, especially in human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) patients (Wittner, 1999). However, occasional cases of microsporidiosis in immunocompetent individuals have been documented (Matsubayashi *et al.* 1959; Ashton & Wirasinha, 1973; Pinnolis *et al.* 1981; Davis *et al.* 1990; Theng *et al.* 2001;). Clinical syndromes associated with human microsporidiosis include intestinal, ocular, pulmonary, renal, urinary, cerebral and disseminated disease (Didier *et al.* 1991a; Weber *et al.* 1993; Kotler & Orenstein, 1998). Commonly identified microsporidia in human patients are *E. intestinalis*, *E. cuniculi*, *E. hellem*, and *Enterocytozoon bieneusi*. Intestinal disease is the most common syndrome caused by microsporidia; clinical symptoms include abdominal pain, chronic diarrhea, and weight loss. *E. bieneusi* followed by *E. intestinalis* are the most prevalent microsporidia causing intestinal disease in man (Kotler & Orenstein, 1998).

Albendazole is the most commonly administered drug used in the treatment of intestinal and disseminated microsporidiosis (Costa & Weiss, 2000). However, the efficacy of this drug is limited. Albendazole therapy for the treatment of microsporidiosis caused by *E. bieneusi* has had poor results. Clinical improvement,

indicated by a decrease in the number of bowel movements, was noted in a few cases by patients receiving albendazole therapy. However, parasite clearance was not observed. Consequently, relapse occurred when therapy was discontinued. Furthermore, in many cases neither clinical nor microbiological improvement was observed (Brasil *et al.* 1998; Georges *et al.* 1998; Leder *et al.* 1998; Goetz *et al.* 2001).

A retrospective study by Leder *et al.* (1998) reviewed the clinical features and therapy of 42 HIV patients with intestinal microsporidiosis. Patients displayed clinical symptoms such as weight loss, diarrhea, abdominal pain, anorexia, and nausea. Patients reported between 1-20 bowel movements per day (average of 4.5) and a decrease in body weight ranging from 2.9 to 48.8 % of initial body weight (average 19 %). All of these patients were infected with either *E. bienewsi* (n = 29) or *E. intestinalis* (n = 13). Of the *E. bienewsi* infected patients, 1 patient had a positive nasopharyngeal aspirate (NPA) while NPA and sputum aspirates from 5 other patients were negative. Sputum and/or bronchial washing, nasal secretions, and NPA samples were obtained from 11 of the *E. intestinalis* infected patients, resulting in evidence of dissemination in 8 of these patients. Metronidazole (400 mg 3 times daily for 1-2 weeks) was administered to 9 of the *E. bienewsi* infected patients and 1 *E. intestinalis* infected patient. Albendazole (400 mg twice daily for 1-2 months) was used in the treatment of 8 of the *E. intestinalis* infected patients and 5 of the *E. bienewsi* infected patients. Five of the *E. bienewsi* infected patients received metronidazole followed by albendazole. None of the *E. bienewsi* treated patients showed clinical improvement. Eight patients reported a decrease in the number of bowel movements per day,

however, in all cases relapse occurred between 6-8 weeks after therapy was discontinued. *E. intestinalis* infected patients responded to albendazole therapy with improvement in diarrhea and/or sinusitis. Clinical relapse was not documented in any of these cases; however, post-treatment samples were not obtained to evaluate parasitic clearance. There was no improvement observed in the *E. intestinalis* infected patient treated with metronidazole (Leder *et al.* 1998).

Albendazole treatment of *Encephalitozoon* spp. infected patients has had better results in reducing clinical symptoms and parasite clearance. However, in some cases complete parasite clearance was not observed and relapses occurred (Molina *et al.* 1995; Dore *et al.* 1995; Gunnarsson *et al.* 1995; Moss *et al.* 1997; Fournier *et al.* 2000). Molina *et al.* (1995) described 5 cases of disseminated microsporidiosis due to *E. intestinalis*. Patients exhibited chronic diarrhea reporting 3-20 bowel movements per day; other symptoms included sinusitis and bronchitis. Patients received 400 mg of albendazole twice daily for between 8-25 days. Clinical improvement was observed in all patients and parasite clearance was observed in the stools of all patients. However, spores were not cleared from the urine of 2 patients, and spores were detected in stool during follow up visits. Diarrhea recurred in these 2 patients (Molina *et al.* 1995). Patients infected with *E. cuniculi* receiving albendazole therapy have also demonstrated clinical improvement, however, parasite clearance was not always observed and clinical relapse was also documented (Fournier *et al.* 2000). Albendazole treatment of dual infection due to *E. hellem* and *Vittaforma corneae* in an AIDS patient complaining of respiratory, abdominal, and urogenital discomfort

resulted in clinical improvement and parasite clearance from respiratory secretions, but not urine (Deplazes *et al.* 1998).

A more effective drug therapy against human microsporidiosis remains to be developed. Identifying enzymes involved in parasitic life cycles is an important step in targeting specific enzymes for potential drug development (McKerrow, 1999). Parasitic proteases have been identified in a variety of protozoa including members of the genera *Plasmodium*, *Trypanosoma*, *Cryptosporidium*, *Giardia*, *Entamoeba*, *Trichomonas*, and *Leishmania* (reviewed by Klemba & Goldberg, 2002). In addition, proteases from these parasites have been investigated at molecular and cellular levels and recognized in host cell invasion, excystation, metabolism, and cytoadherence, and also as virulence factors (Klemba & Goldberg, 2002).

Aminopeptidases are a group of proteases that cleave amino acid residues from the N-terminus of peptide substrates and proteins. Aminopeptidases have been identified in a number of different organisms and are involved in essential physiologic functions (Taylor, 1993). Aminopeptidases of opportunistic pathogens have been described in facilitating the attachment and invasion of bacteria to host cells, nutrient uptake, dissemination of infection (Miyoshi & Shinoda, 2000), and *Cryptosporidium parvum* excystation, a critical life cycle event (Okhuysen *et al.* 1996).

Inhibitors of parasitic aminopeptidases have been evaluated as potential chemotherapeutic agents of parasitic diseases (Okhuysen *et al.* 1996; Day & Chen, 1998). Most aminopeptidases are inhibited by bestatin and/or its analogs, and metallopeptidases (aminopeptidases that require at least one metal ion for catalytic

activity) are additionally inhibited by chelating agents such as ethylenediamine tetraacetate (EDTA) (Taylor, 1993). Aminopeptidase activity of enzyme extracts of *Leishmania* spp. and *Toxoplasma gondii* are strongly inhibited by bestatin, EDTA and 1,10-phenanthroline (Berthonneau *et al.* 2000; Morty & Morehead, 2002). The aminopeptidase inhibitors bestatin and nitrobestatin successfully inhibited aminopeptidase activity and parasite growth of *Plasmodium chabaudi chabaudi* and *Plasmodium falciparum in vitro* (Nankya-Kitaka *et al.* 1998). Also, an arginine aminopeptidase of *C. parvum* oocysts was successfully inhibited *in vitro* (Okhuysen *et al.* 1994). This aminopeptidase exhibited highest activity during excystation *in vitro*, and enzymatic activity and excystation were successfully inhibited with the aminopeptidase inhibitor amastatin, EDTA, or 1,10-phenanthroline (Okhuysen *et al.* 1994; Okhuysen *et al.* 1996).

Aminopeptidase activity of *E. intestinalis*, *E. cuniculi*, *E. hellem*, and *V. cornea* has been characterized using a variety of fluorometric substrates (Millership *et al.* 2001; Millership *et al.* 2002b). The aminopeptidase described showed highest

activity against the substrate leucine and demonstrated maximal activity at 37 °C and pH 7.2. The aminopeptidase inhibitors, bestatin and nitrobestatin, and the chelating agent, 1,10-phenanthroline, successfully inhibited the enzymes of all 3 microsporidia (Millership *et al.* 2002a). Visualization of aminopeptidase activity using L-Met-7-amino-4-trifluoromethyl coumarin (L-Met-AFC) substrate was associated with parasites in the host cell cytoplasm and the microsporidial polar tubule. The molecular masses of the enzymes, estimated using fluorogenic analysis with L-Met-AFC on polyacrylamide gels, were 70, 74, 72, and 78 kDa for *E. intestinalis*, *E. cuniculi*, *E. hellem*, and *V. corneae*, respectively (Millership *et al.* 2002a).

Aminopeptidase inhibitors have been evaluated both *in vitro* and *in vivo* as antimicrosporidial therapies. *In vitro* studies using bestatin, amastatin, and nitrobestatin significantly reduced *E. cuniculi* replication without significant host cell toxicity (Millership *et al.* 2001). *In vivo* experiments showed that *E. cuniculi* infected athymic mice showed prolonged survival when treated with bestatin (Millership *et al.* 2001).

In this work, the cloning, sequencing, and molecular characterization of an aminopeptidase gene from 3 isolates of *Encephalitozoon* are described.

Material and Methods

Encephalitozoon Isolates

The bird isolate of *E. hellem* was isolated from droppings of a love bird (*Agapornis roseicollis*) (Snowden *et al.* 2000). The human *E. hellem* isolate was from a human AIDS patient suffering from keratoconjunctivitis (Didier *et al.* 1991a). The *E. cuniculi* isolate was originally from a rabbit (*Oryctolagus cuniculi*) (Shaddock, 1969). All isolates were kindly provided by Dr. Karen Snowden, Texas A&M University.

Spore Collection

Cryopreserved pellets of the bird and human *E. hellem* isolates and *E. cuniculi* were thawed and maintained in culture in 75 cm² flasks using RK-13 rabbit kidney cells (ATCC no. CCL-37) in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, St. Louis, MO) supplemented with L-glutamine (2 mM), 10 % heat inactivated fetal bovine serum (FBS) and antibiotic/antimycotic (Gibco BRL, Grand Island, NY) at 37 °C and a humidified 5 % CO₂ in air atmosphere. Spores were collected periodically from the culture supernatants. Spores were pelleted by centrifugation at 800 *g* for 20 min and washed with sterile tap H₂O to lyse host cells. The final spore pellets were resuspended in 5 ml DMEM. Samples of the resuspended spore pellets were quantified on a hemocytometer, and the spore suspensions were adjusted to 1.0 x 10⁹ spores/ml and stored at 4 °C until used. Two aliquots of 1.0 x 10⁹ spores of each isolate described above, *E. cuniculi* and *E. hellem* (human and bird), were placed in 15

ml conical tubes and washed twice in phosphate-buffered saline (PBS). The final spore pellets were transferred to 2 ml bead-beater tubes and centrifuged at 800 *g* for 5 min and the remaining supernatant discarded. One tube of spores for each isolate was used for total RNA isolation and the other frozen at -20 °C for later isolation of genomic DNA (gDNA).

Total RNA Isolation

The washed spore pellets, in each 2 ml microtube, were resuspended in 750 µl lysis/binding buffer (RNAqueous, Ambion, Austin, TX) and approximately 600 µl 0.5 mm zirconia/silica beads (Biospec Products, Bartlesville, OK) was added to each tube. Spores were beaten at 500 rpm for 30 s in a Mini-bead beater (Biospec Products, Bartlesville, OK), then cooled on ice for 1 min. This was repeated 4 times. The tubes were centrifuged for 2 min at 5600 *g* and the supernatants collected. An additional 600 µl of lysis buffer was added to each tube and the above cycle repeated. The tubes were centrifuged again for 2 min at 5600 *g* and the supernatants collected. The supernatants from each isolate were pooled and total RNA was purified from each according to the RNAqueous kit protocol (Ambion).

Genomic DNA Isolation

Genomic DNA was isolated from the frozen spore samples of *E. cuniculi* and bird and human *E. hellem* isolates described above. The samples were thawed at 37 °C and approximately 600 µl 0.5 mm zirconia/silica beads (Biospec Products) and 180

μl lysis solution (GENE Elute, Sigma, St. Louis, MO) were added to pellets. Spores were bead blasted as described above. Each supernatant was treated separately with Proteinase K and DNA eluted following manufacturer's instructions (GENE Elute).

cDNA First Strand Synthesis

E. hellem bird and human isolate and *E. cuniculi* isolate cDNAs were prepared from the total RNA described above (SMARTTMRACE cDNA amplification kit; Clontech Laboratories, Palo Alto, CA). First strand 3' cDNA was prepared by mixing 3 μl RNA sample and 1 μl 3'-CDS primer, and first strand 5' cDNA was prepared by mixing 3 μl RNA sample, 1 μl 5'-CDS primer, and 1 μl SMART II oligo. Sterile H₂O was added to each reaction to a final volume of 5 μl. The reaction mixes were incubated at 70 °C for 2 min in a heated block and briefly cooled on ice for an additional 2 min. Next, to each tube 2 μl 5X First-strand buffer, 1 μl DTT (20 mM), 1 μl dNTP Mix (10 mM), and 1 μl MMLV reverse transcriptase (200 units/μl) were added and the reactions mixed. The reactions were incubated for 1.5 h at 42 °C in an air incubator. Following this step, 20 μl of Tricine-EDTA buffer was added to each tube and the tubes were heated at 72 °C in a heated block for 7 min. Samples were stored at -20 °C until used.

Table 2.1. Oligonucleotide primers used for amplification and sequencing reactions. Primers 1 and 2 were used for homology PCR with gDNA from both *E. hellem* and *E. cuniculi*. Primers 3 -14 are *E. hellem* specific primers and Primers 15-26 are specific for *E. cuniculi*.

Primer Number	Primer Name	Sequence 5' – 3'	Direction	Position
1	ZAPFEc	CAN CAY TTY GAR CCN CAN GA	Forward	436-455
2	ZAPREc	CCA NAR RTC RTC CCA CCA	Reverse	1019-1036
3	ZAPEhF	GCG GGA GGA GTA TGG AGA CAG G	Forward	576-597
4	ZAPEhR	CTG TCT CCA TAC TCC TTC CGC AGC G	Reverse	572-596
5	ZAPEh5F	GCG ATG GAG AAC TGG	Forward	874-888
6	ZAPEhA	GAG CAA GCA TCA TCA GG	Forward	1256-1272
7	ZAPEh3R300	ATT CTT CTG CAC CAT CG	Reverse	2378-2394
8	ZAPEh5F1090	GCA TGT GGA TTG TGC C	Forward	1526-1541
9	ZAPEh5°F	GTA TGA AGT GGA TTG AAT TG	Forward	-2-18
10	ZAPEh3°R	CAG AGG AGC AGA GTC CTG C	Reverse	2575-2593
11	ZAP350F	GGA TAC ATT GTG GGA GAG	Forward	352-369
12	ZAP690R	GAC TTG CTC CAG	Reverse	681-692
13	ZAP1857F	GAG AAG CTG ATG CGG	Forward	1852-1866
14	ZAP2169R	CCC CAA TCA GGT GCT C	Reverse	2149-2163
15	ZAPEC5PF	AAA TGA GGT GGA TTA AAG TAA TGG C	Forward	-2-23
16	ZAPEC3PR	TTA CTT CTC AGC CTC CGG C	Reverse	2576-2595
17	ZAPEC5PFN	TTG AGA GGT ATG TCG GCG AG	Forward	1288-1269
18	ZAPEC3PRN	TGC ACA GAC ACA ACA GGA TAC C	Reverse	1431-1452
19	ZAPEC5PFN2	AGG GCT TCT GCC CAT GA	Forward	25-41
20	ZAP ECR1900	ATC TCA AGC TCC TTG GC	Reverse	211-227
21	ZAPEC3PRN2	AGA AGC CCA CGA AGG ACC	Reverse	2560-2577
22	ZAPEC3PRN3	AGA AGC CCA CGA AGG ACC TCC TCT C	Reverse	2553-2577
23	ZAPEc5PN4	CCG GCG GTA CAT CAA GGA GCA C	Forward	1309-1330
24	ZAPECF1800	TCG TAC CAT GTT GCT CG	Forward	1816-1832
25	ZAPEcS1-R	TCG CCT TCA TAT CGG	Reverse	489-503
26	ZAPECS2F	TCG ACA GCA TCA GCT AC	Forward	1817-1833

Homology PCR

Degenerate primers, Primers 1 and 2 (Table 2.1; Figure 2.1 on p. 36), were designed from conserved regions of the available aminopeptidase sequence of *E. cuniculi* (GenBank accession no. CAA06646) (Fig. 2.1) for use in homology PCR. Advantage 2 PCR Enzyme System (Clontech Laboratories, Inc., Palo Alto, CA) reagents were used in all PCR reactions, which were carried out in a PCR Express or Sprint thermocycler (Hybaid, Ashford, Middlesex, TW15 1XB). [The sequences and location of all primers described throughout the text can be found in Table 2.1.]

The accuracy of Primers 1 and 2 was tested in a PCR reaction containing 1 µl (50-100 ng) *E. cuniculi* gDNA with 15.5 µl dH₂O, 2.5 µl 10X cDNA PCR Reaction buffer, 0.5 µl dNTP Mix (10 mM), 0.5µl Advantage 2 Polymerase Mix (50X), 2.5 µl Primer 1 (0.1 µM), and 2.5 µl Primer 2 (0.1 µM) performed under the following cycling parameters: initial denaturation, 94 °C for 1 min; 40 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 30 s, end extension at 72 °C for 1 min 30 s; a final extension at 72 °C for 10 min, and 4 °C hold. The PCR product was visualized by separation on a 1 % high melt agarose gel (W/V) in TBE buffer (45 mM Tris, 45 mM borate, 1mM EDTA, pH 8.0) followed by ethidium bromide staining and UV transillumination.

Multiple amplicons were present, so the remainder of the PCR product was separated on a 1 % low melt agarose gel (W/V) in TAE buffer (45 mM Tris, 45 mM acetic acid, 1 mM EDTA, pH 8.0) and a 600 bp band cut from the gel.

The gel slice was heated at 72 °C for 10 min and ligated into plasmid vector PCRTM II and competent *Escherichia coli* transformed (TOPO-TA Cloning Kit, Invitrogen, San Diego, CA). Plasmid DNA from each color-selected clone was purified from overnight Luria Broth (LB) cultures by a modified alkaline lysis procedure (QIAprep Spin Miniprep Kit; Qiagen®, Valencia, CA). Two clones were selected and sequencing was carried out in either an ABI PRISM Model 373A or ABA Model 377 automated sequencer with Version 1.2 or Version 2.1 software, respectively (Gene Technologies Laboratory, Institute of Developmental and Molecular Biology, Department of Biology, Texas A&M University, College Station, TX). The cloned inserts were sequenced using M13 F and M13 R primers. These primer sites are located within the plasmid vector flanking the PCR insert. The obtained nucleotide sequences were then subjected to BLAST searches (Altschul *et al.* 1992) by the GenBank database (National Center for Biotechnology Information, National Institute of Health).

The same PCR reaction mix and cycling parameters as described above were performed again except 1 µl (50-100 ng) *E. hellem* (bird) gDNA was used in lieu of *E. cuniculi* gDNA. A single 600 bp amplicon was obtained and 4 µl of PCR product was ligated into a plasmid vector as described above. Plasmid DNA was isolated and 2 clones sequenced using primers M13 F and M13 R. The obtained nucleotide sequences were also subjected to BLAST searches. Specific *E. hellem* primers, Primer 3 and Primer 4, were designed from the obtained sequence for use in RACE reactions (Table 2.1 and Fig. 2.2 on p. 36).

Amplification of Aminopeptidase Genes from cDNA

3'RACE E. hellem (bird)

The 3' RACE PCR reaction was performed using 1.2 µl of a 1:2 dilution of *E. hellem* (bird) 3' first strand cDNA and 16.8 µl dH₂O, 2.5 µl 10X cDNA PCR Reaction buffer, 0.5 µl dNTP Mix (10 mM), 0.5 µl Advantage 2 Polymerase Mix (50X), 2.5 µl Primer 3 (0.1 µM), and 2.5 µl of 10X Universal Primer Mix (UPM; primer complementary to the linker added to the 3' end of the 3' cDNA during synthesis) under the following cycling parameters: 40 cycles of denaturation at 94 °C for 5 s, annealing at 68 °C for 10 s, end extension at 72 °C for 3 min; final extension at 72 °C for 10 min, and 4 °C hold. A 2000 bp amplicon was ligated and transformed as described above. Plasmid DNA was extracted and selected clones sequenced with M13 F, M13 R, and Primers 5, 6, 7, 8, and 12 (Table 2.1).

5'RACE E. hellem (bird)

A primary 5'RACE PCR reaction was performed as the 3' RACE PCR reaction except 5' first strand cDNA replaced 3' first strand cDNA, and Primer 2 was used in place of Primer 3. A nested PCR followed this reaction using 1.0 µl of a 1:100 dilution of the primary PCR product and 17.0 µl dH₂O, 2.5 µl 10X cDNA PCR Reaction buffer, 0.5 µl dNTP Mix (10 mM), 0.5 µl Advantage 2 Polymerase Mix (50X), 2.5 µl Primer 4 (0.1 µM), and 2.5 µl of 10X Nested Universal Primer (NUP; nested primer complementary to the linker added to the 3' end of the 3' cDNA during synthesis). The cycling parameters were as previously described for 25 cycles. An

approximately 600 bp band was excised from a 1 % low melt agarose gel (W/V) in TAE and subsequently ligated as previously described. Plasmid DNA was extracted and selected clones sequenced with primers M13 F and M13 R.

The obtained nucleotide sequences from the 3' and 5' RACE reactions were aligned using SEQUENCHER (Gene Codes Corp, Ann Arbor, MI) and used to design 5'- and 3'-end specific primers, Primers 9 and 10 (Table 2.1), for amplifying the *E. hellem* (human) aminopeptidase gene.

Amplification Aminopeptidase genes from E. hellem (human) cDNA

Two PCR reactions were performed to obtain overlapping fragments encompassing the entire *E. hellem* (human) aminopeptidase gene. The first PCR reaction was performed using 2.5 µl of *E. hellem* (human) 3' first strand cDNA and 15.5 µl dH₂O, 2.5 µl 10X cDNA PCR Reaction buffer, 0.5 µl dNTP Mix (10 mM), 0.5 µl Advantage 2 Polymerase Mix (50X), 2.5 µl Primer 2 (0.1 µM), and 2.5 µl of Primer 9 (0.1 µM) under the following cycling parameters: 25 cycles of denaturation at 94 °C for 5 s, annealing at 68 °C for 10 s, and extension at 72 °C for 4 min; final extension at 72 °C for 10 min, and 4 °C hold. The PCR product was purified (QIAgen PCR Purification Kit, QIAGEN) and a 1000 bp amplicon was ligated and transformed as described above. Plasmid DNA was extracted and selected clones sequenced with Primers 3, 12 and M13 F and M13 R. The second PCR reaction contained 2.5 µl of *E. hellem* (human) 5' first strand cDNA and 15.5 µl dH₂O, 2.5 µl 10X cDNA PCR Reaction buffer, 0.5 µl dNTP Mix (10 mM), 0.5 µl Advantage 2 Polymerase Mix (50X), 2.5 µl Primer 3 (0.1 µM), and 2.5 µl of Primer 10 (0.1 µM) under the following

cycling parameters: 25 cycles of denaturation at 94 °C for 5 s, annealing at 68 °C for 10 s, and extension at 72 °C for 4 min; final extension at 72 °C for 10 min, and 4 °C hold. The PCR product was purified and a 2000 bp amplicon was ligated and transformed as described above. Plasmid DNA was extracted and selected clones sequenced with M13 F, M13 R and Primers, 5, 6, 8, and 10 (Table 2.1).

Amplification of Aminopeptidase Genes from gDNA

E. hellem bird and human isolates

The full length aminopeptidase gene was directly amplified from *E. hellem* bird and human gDNA. The 25 µl reaction mixes contained 17.0 µl dH₂O, 2.5 µl 10X cDNA PCR Reaction buffer, 0.5 µl dNTP Mix (10 mM), 0.5 µl Advantage 2 Polymerase Mix (50X), 2.5 µl Primer 9 (0.1 µM), and 2.5 µl of Primer 10 (0.1 µM), and either 1 µl (50-100 ng) *E. hellem* (bird) or *E. hellem* (human) gDNA. Touch down PCR was performed as follows: initial denaturation at 94 °C for 2 min; 20 cycles of 94 °C for 10 s, 65 °C for 10 s, 72 °C for 3 min; 20 cycles of denaturation at 94 °C for 10 s, annealing at 55 °C for 10 s, and extension at 72 °C for 3 min; final extension for 10 min at 72 °C, and 4 °C hold. The amplicons were visualized by ethidium bromide stained agarose gels as described above. Repeated attempts to clone the products for sequencing were not successful. PCR products from several reactions for each *E. hellem* isolate were pooled and purified (QIAGEN PCR Purification Kit, QIAGEN). The purified product was visualized on an agarose gel and quantitated.

The purified PCR products were directly sequenced with Primers 3, 5, 6, 8, 10, 11, 12, 13 and 14.

E. cuniculi

The targeted aminopeptidase gene of *E. cuniculi* was amplified from gDNA using a nested PCR protocol, since a single usable band was not obtained in PCR reactions with 5' and 3' primers. Specific primers were designed from *E. cuniculi* aminopeptidase nucleotide sequence available in the GenBank database (accession no. AJ005644) and used to generate overlapping fragments that were subsequently cloned and sequenced.

The primary PCR was performed using 1.0 µl (50-100 ng) of *E. cuniculi* gDNA, 15.5 µl dH₂O, 2.5 µl 10X cDNA PCR Reaction buffer, 0.5 µl dNTP Mix (10 mM), 0.5 µl Advantage 2 Polymerase Mix (50X), 2.5 µl Primer 15 (0.1 µM), and 2.5 µl of Primer 16 (0.1 µM) under the following cycling parameters: initial denaturation at 94 °C for 3 min; 30 cycles of denaturation at 94 °C for 45 s, annealing at 62 °C for 30 s, and extension at 72 °C for 3 min; final extension at 72 °C for 10 min and 4 °C hold.

The nested PCR reaction for the 5' end of the gene was performed using 2.0 µl of primary PCR product, 14.5 µl dH₂O, 2.5 µl 10X cDNA PCR Reaction buffer, 0.5 µl dNTP Mix (10 mM), 0.5 µl Advantage 2 Polymerase Mix (50X), 2.5 µl Primer 18 (0.1 µM), and 2.5 µl of Primer 19 under the following cycling parameters: initial denaturation at 94 °C for 3 min; 40 cycles of denaturation at 94 °C for 45 s, annealing at 62 °C for 30 s, and extension at 72 °C for 2 min; final extension at 72 °C for 10 and 4

°C hold. The PCR products were purified (QIAGEN PCR Purification Kit, QIAGEN) and the final product was visualized on a 1% agarose gel (W/V) in TBE buffer. Approximately 100 ng PCR product was sequenced with Primer 5. In addition, 4 µl of the purified PCR product was cloned and sequenced using M13 R and Primers 19, and 20.

The 3' end was generated by PCR using 2.0 µl of a 1:5 dilution of *E. cuniculi* gDNA, 15.5 µl dH₂O, 2.5 µl 10X cDNA PCR Reaction buffer, 0.5 µl dNTP Mix (10 mM), 0.5 µl Advantage 2 Polymerase Mix (50X), 2.5 µl Primer 22 and 2.5 µl of Primer 23 (0.1 µM) under the following cycling parameters: initial denaturation of 94 °C for 3 min; 40 cycles of 94 °C for 45 s, and 72 °C for 2 min; final extension at 72 °C for 10 and 4 °C hold. The resulting amplicon was cloned and sequenced with Primers 21, 23, 25 and 26.

Sequence Alignment Comparisons

The nucleotide and predicted amino acid sequences of the zinc metallo-aminopeptidase (ZAP) genes obtained in the study were aligned and compared using ClustalW, EMBL-EMBI, European Bioinformatics Institute, <http://www.ebi.ac.uk/clustalw>). The gDNA and cDNA sequences for each *E. hellem* isolate were aligned and compared; the gDNA and cDNA sequences were also compared between the bird and human isolates. The predicted amino acid sequences of both *E. hellem* isolates obtained from cDNA were aligned and compared with the amino acid sequence predicted from the *E. cuniculi* gDNA sequence. In addition, the *E.*

cuniculi gDNA sequence was aligned and compared to the *E. cuniculi* ZAP sequence in GenBank (accession no. CAA06446).

Protein Motif Blast Search and Protein Mass Prediction

The aminopeptidase predicted amino acid sequences of *E. hellem* human, *E. hellem* bird and *E. cuniculi* were subjected to protein motif searches using Prosite, ExPASy, Swiss Institute of Bioinformatics (SIB), and the predicted mass was calculated using Peptide Mass, ExPASy, SIB (<http://us.expasy.org/tools/peptide-mass.html> and <http://us.expasy.org/>).

In addition the GenBank sequences for *E. cuniculi* zinc metalloprotease, glutamyl and leucine aminopeptidases (GenBank accession nos. CAA06646, NP_597136, NP_586294) were also subjected to protein motif searches using Prosite and ExPASy (SIB), and the predicted mass was calculated using Peptide Mass, ExPASy (SIB).

Results

Homology PCR and Aminopeptidase Gene Acquisition

Homology PCR to acquire an aminopeptidase gene fragment from *E. cuniculi* generated a 600 bp amplicon that was successfully ligated and cloned. The resulting sequence was subjected to a BLAST search and shared 100 % identity with *E. cuniculi* aminopeptidase (GenBank accession no. CAA06646). *E. hellem* homology PCR also generated a 600 bp amplicon that was successfully cloned and sequenced. A BLAST search of the obtained sequence showed a 71 % identity shared with *E. cuniculi* aminopeptidase (GenBank accession no. CAA06646) and glutamyl aminopeptidase (GenBank accession nos. NP586759 and CAD24886). The sequence matched the *E. cuniculi* aminopeptidase sequences more closely than any other sequences in the database. *E. hellem* specific primers, Primer 3 and Primer 4 (Table 2.1), were designed from the obtained 600 bp sequence and used to obtain the full length aminopeptidase genes from *E. hellem* (bird) cDNA.

E. hellem (bird) 3' and 5' RACE reactions generated approximately 2000 bp and 600 bp amplicons, respectively, that were successfully cloned and sequenced. The full length aminopeptidase gene was obtained by aligning the overlapping sequences and included the start methionine codon, polyadenylation signal and stop codon. The open reading frame was 2598 bp and 865 predicted amino acids (Fig. 2.1 and Fig. 2.2). From this sequence *E. hellem* specific 5' and 3' primers, Primers 9 and 10 (Table 2.1), were designed to amplify the aminopeptidase gene sequences from *E. hellem* (human) cDNA and *E. hellem* (bird and human) gDNA. The open reading frame was 2995 bp and 864 predicted amino acids and included the start methionine but lacked the stop codon. The aminopeptidase gene sequence of *E. cuniculi* was successfully obtained from gDNA using a nested PCR protocol that produced 2 overlapping fragments yielding a gene of approximately 2600 bp with a predicted amino acid sequence of 863 residues (Figs. 2.1 and 2.2).

EH	<u>ATGAAGTGGATTGAATTGATGATTGGATTGATGCCCATGATTGAAGGCAGAAAAATGGAA</u>	60
Mu5	<u>ATGAAGTGGATTGAATTGATGATTGGATTGATGCCCATGATTGAAGGCAGAAAAATGGAA</u>	60
	PRIMER 9	
EH	GGAAAGCAGTGGGACAGAAATTTCTCTCGAAGGCTGGACAGGACTGTTGTTCTTCGCACTAC	120
Mu5	GGAAAGCAGTGGGACAGAAATTTCTCTCGAAGGCTGGACAGGACTGTTGTTCTTCGCACTAC	120
EH	GACCTGCATGTGAAGGTGATGGACACGGGGTTCTGCGGAAACGTAGGGATCCGGGTGGAT	180
Mu5	GACCTGCATGTGAAGGTGATGGACACGGGGTTCTGCGGAAACGTAGGGATCCGGGTGGAT	180
EH	ATCTCGCAGCCTGTGGATGAGATAATGATGAATGCAAAAGGAGCTTGACGTGCTGAGTGCA	240
Mu5	ATCTCGCAGCCTGTGGATGAGATAATGATGAATGCAAAAGGAGCTTGACGTACTGAGTGCA	240
EH	GGGATTGAGATTGATGGAAGGAGGATTGATGGAGAGGTGGTTCATGGGAGACGACGAGAAAG	300
Mu5	GGGATTGAGATTGATGGAAGGAGGATTGATGGAGAGGTGGTTCATGGGAGACGACGAGAAAG	300
EH	GAGTACGAGGTTGTAAGGATCAGGTTTCCCTCGACGAYACCCAAGGGAGAAAGGATACATT	360
Mu5	GAGTACGAGGTTGTAAGGATCAGGTTTCCCTCGACGATACCCAAGGGAGAAAGGATACATT	360
	PRIMER 11	
EH	GTGGGAGAGTTCTGCGGAGACTACAACCTCTGGTCTGGTTGGGTTTTACAAGAGCGGGGGA	420
Mu5	GTGGGAGAGTTCTGCGGAGACTACAACCTCTGGTCTGGTTGGGTTTTACAAGAGCGGGGGA	420
EH	GAGTCGAAGGTCTACAGCACACACTTTGAGCCGACGGATGCAAGAAGGGTGTTTCCATGC	480
Mu5	GAGTCGAAGGTCTACAGCACACACTTTGAGCCGACGGATGCAAGAAGGGTGTTTCCATGC	480
EH	TTTGACCAAGCCGATATGAAGGCAACGTTTAGCATTTTCGATTGATGCGCCGTCAAACCTT	540
Mu5	TTTGACCAAGCCGATATGAAGGCAACGTTTAGCATTTTCGATTGATGCGCCGTCAAACCTT	540
EH	GTTGTTCTGTGCAACAGCCCCCTCTGTGCCTTCGCTGCGGAAGGAGTATGGAGACAGGGCG	600
Mu5	GTTGTTCTGTGCAACAGCCCCCTCTGTGCCTTCGCTGCGGAAGGAGTATGGAGACAGGGCG	600
	PRIMERS 3 & 4	
EH	ATTGAGTACTTCACGAAGACAAAGCAAGATGTCTACATATCTTGTGTCATTGTTGCTGGC	660
Mu5	ATTGAGTACTTCACGAAGACAAAGCAAGATGTCTACATATCTTGTGTCATTGTTGCTGGC	660
EH	GAGCTTGAGTACATTGAGGACTGGAGCAAGTCTGGAGTGAGGCTAAGGGTGATGGACAG	720
Mu5	GAGCTTGAGTACATTGAGGACTGGAGCAAGTCTGGAGTGAGGCTAAGGGTGATGGACAG	720
	PRIMER 12	
EH	GGGAAGGAAGAGGCGTCGTGGGGAAAGGTATGGGCTTGAGGTTGCAAAAGAGATGCATCGAC	780
Mu5	GGGAAGGAAGAGGCGTCGTGGGGAAAGGTATGGGCTTGAGGTTGCAAAAGAGATGCATCGAC	780

Fig. 2.1. Nucleotide aminopeptidase sequence alignment of *E. hellem* human (EH) and bird (Mu5) isolates obtained from cDNA. The nucleotides underlined represent primer locations, bolded nucleotides differ between bird and human isolates with no change in the predicted amino acid sequences, nucleotides bolded and underlined differ between sequences and differ in the predicted amino acid sequence. The methionine start codon is triple underlined and the polyadenylation signal is double underlined.

EH TACTTTGAGGAGTACTTTGGGATTGAGTATGAGTTTCCTGAGAAGGGGATGGCAAAGATA 840
 Mu5 TACTTTGAGGAGTACTTTGGGATTGAGTATGAGTTTCCTGAGAAGGGGATGGCAAAGATA 840

EH GACATGATTGGGATTCCGAGCTTTGGCAGTGGAGCGATGGAGAACTGGGGGCTCATTACG 900
 Mu5 GACATGATTGGGATTCCGAGCTTTGGCAGTGGAGCGATGGAGAACTGGGGGCTCATTACG 900

PRIMER 5

EH TTCCGAAAGGAGAGTCTTCTGTACTCTGATGGAAAGGACAGTGTGAGCAAAAGAAGAA 960
 Mu5 TTCCGAAAGGAGAGTCTTCTGTACTCTGATGGAAAGGACAGTGTGAGCAAAAGAAGAA 960

EH GTAGCTGAAACTGTGTGCCACGAGCTGGGGCACATGTGGTTTGGAAACCTTGTGACGATG 1020
 Mu5 GTAGCTGAAACTGTGTGCCACGAGCTGGGGCACATGTGGTTTGGAAACCTTGTGACGATG 1020

EH GAGTGGTGGGACGACCTTTGGCTGAACGAGGGATTTGCAACATGGATATCGTTCAAGGGG 1080
 Mu5 GAGTGGTGGGACGACCTTTGGCTGAACGAGGGATTTGCAACATGGATATCGTTCAAGGGG 1080

EH ATGTCTGAGATAGGAAAGGAGTATGTTGACTGGGATGTCCAGGGAGAGTTTGTGCTGTGG 1140
 Mu5 ATGTCTGAGATAGGAAAGGAGTATGTTGACTGGGATGTCCAGGGAGAGTTTGTGCTGTGG 1140

EH AATGTGATACGGGGGATGCTTGAGGATGGGCTTGGAAGAGCCACAAGATCCGGATGGAG 1200
 Mu5 AATGTGATACGGGGGATGCTTGAGGATGGGCTTGGAAGAGCCACAAGATCCGGATGGAG 1200

EH GTTTCCGATCCTGGAGAGATTGGAGAGATCTTTGATGCGATCAGCTACAGCAAAGGAGCA 1260
 Mu5 GTTTCCGATCCTGGAGAGATTGGAGAGATCTTTGATGCGATCAGCTACAGCAAAGGAGCA 1260

EH AGCATCGTCAGGATGGTTGAGAAGTATGTTGGGGAGTCTGTATTTATGTCTGGGATCCGG 1320
 Mu5 AGCATCATCAGGATGGTTGAGAAGTATGTTGGGGAGTCTGTATTTATGTCTGGGATCCGG 1320

PRIMER 6

EH CGATACATCAAGGAGCACATGTATGGGAACGGGAATGGAATGAGCCTGTGGAAGGCGATT 1380
 Mu5 CGATACATCAAGGAGCACATGTATGGGAACGGGAATGGAATGAGCCTGTGGAAGGCGATT 1380

EH GGGGAGTCCTATGGAAAGGACATTTCTGTCGATGGTTGAGGGATGGATTTTCGAGGCAGGA 1440
 Mu5 GGGGAGTCCTATGGAAAGGACATTTCTGTCGATGGTTGAGGGATGGATTTTCGAGGCAGGA 1440

EH TACCCTGTGATAAGTGTCAGCGAGAGTGGGGAGAACCTCGTTCTATCGCAGAGGAGATAC 1500
 Mu5 TACCCTGTGATAAGTGTCAGCGAGAGTGGGGAGAACCTCGTTCTATCGCAGAGGAGATAC 1500

EH TCGATGCTTGGAAGGACGACAGCATGTGGATTGTACCTGTTGTGATAACATGGGGG 1560
 Mu5 TCGATGCTTGGAAGGACGACAGCATGTGGATTGTGCTGCTGTTGTGATAACATGGGGG 1560

PRIMER 8

EH TCTGGAGAGAACGAAAGCATTGAGGTTGGAGAGAGAGAGGTTGTGATTCCACGGAGGAGC 1620
 Mu5 TCTGGAGAGAACGAAAGCATTGAGGTTGGAGAGAGAGAGGTTGTGATTCCACGGAGGAGC 1620

EH GATGTGTACAAGGTGAACAGCTCGTATGCAGGGTTCTACCGGGTGCTGTACGAGGCCGAT 1680
 Mu5 GATGTGTACAAGGTGAACAGCTCGTATGCAGGGTTCTACCGGGTGCTGTACGAGGCCGAT 1680

EH GCACTGAGAAGGCTTGAGGAGAGGCTTGAGGAGATGGGCGACATGGACCGAGTGAACATG 1740
 Mu5 GCACTGAGAAGGCTTGAGGAGAGGCTTGAGGAGATGGGCGACATGGACCGAGTGAACATG 1740

Fig. 2.1 continued.

EH	GTCTAGGATGTGTTTGGGCTTGGATTTGGACTGTATGGAGGGATTGGAGAGAGGCTCCGG	1800
Mu5	GTCTAGGATGTGTTTGGGCTTGGATTTGGACTGTATGGAGGGATTGGAGAGAGGCTCCGG	1800
EH	AAGATAGGGGAGTACTACTCTGACGGATACTGTGTTGCCCGAAGCACGGTTGAGAAGCTG	1860
Mu5	AAGATAGGGGAGTACTACTCTGACGGATACTGTGTTGCCCGAAGCACGGTTGAGAAGCTG	1860
PRIMER 13		
EH	ATGCGGATCCGGAGTGTGTTCTACGACGATGCCGAGATTGTGTCTGCGGTTGACAAGCGA	1920
Mu5	ATGCGGATCCGGAGTGTGTTCTACGACGATGCCGAGATTGTGTCTGCGGTTGACAAGCGA	1920
EH	GTTCTGGGGCTGATTGAGGAGAGGATTGAGTCTATTGACACCTCTGTGTGTGAGACGTCT	1980
Mu5	GTTCTGAGGCTGATTGAGGAGAGGATTGAGTCTATTGACACCTCTGTGTGTGAGACGTCT	1980
EH	GTGGAAGGATCAGCATGAACAAGTACCTGCTGTCTGTTGGCATTGAGGTTGGAAGTGCC	2040
Mu5	GTGGAAGGATCAGCATGAACAAGTACCTGCTGTCTGTTGGCATTGAGGTTGGAAGTGCC	2040
EH	TCTGCGATGAGGAAGGTGTCTGAGCTGTGGAGGCGGCACGTGAGGAGGGAGCGGAGCTT	2100
Mu5	TCTGCGATGAGGAAGGTGTCTGAGCTGTGGAGGCGGCACGTGAGGAGGGAGCGGAGCTT	2100
EH	GGAGAACTTCGGTGGATTGTGTACAAGGGAGTTGTTGACGACAACCTTGAGCACCTGATT	2160
Mu5	GGAGAACTTCGGTGGATTGTGTACAAGGGAGTTGTTGACGACAACCTTGAGCACCTGATT	2160
PRIMER 14		
EH	GGGGAGTACAAGAGTGGAAAGGAGGCCTGGAAGCCGATGGGAGGTGATAAAATGGGTTTCAGC	2220
Mu5	GGGGAGTACAAGAGTGGAAAGGAGGCCTGGAAGCCGATGGGAAAGTATAAATGGGTTTCAGC	2220
EH	GGGATAAAGAGGAAGGAGAGCTTTGCAAGAGGTTGTGAAGAGGCTGTGCGAGTTTCAGTGTT	2280
Mu5	GGGATAAAGAGGAAGGAGAGCTTTGCGAGAGGTTGTGAAGAGGCTGTGCGAGTTTCAGTGTT	2280
EH	GAGGACATTGGTGTGGTGATAGCAATAATCTGCAGAAAGCGGGAACCTTCCGTGACGAGATG	2340
Mu5	GAGGACATTGGTGTGGTGATAGCAATAATCTGCAGAAAGCGGGAACCTTCCGTGACGAGATG	2340
EH	GTTGAGTATGTTGTGTGTCGACCCGGGAGGAGCTTTATTGATGGTGCAGAAAGATGTGATG	2400
Mu5	GTTGAGTATGTTGTGTGTCGACCCGGGAGGAGCTTTATTGATGGTGCAGAAAGATGTGATG	2400
PRIMER 7		
EH	CTATACAACACCATTTGTGATGTCTCTCCGGCATGTGAGCAGTGACCCAATGGTGGAGAAA	2460
Mu5	CTATACAACACCATTTGTGATGTCTCTCCGGCATGTGAGCAGTGACCCAATGGTGGAGAAA	2460
EH	GTGTCTGAGTTCCTTTCTGGGATCAAGCACTCTGGAAGCAGTCTGAGCATTGAGAAGGTG	2520
Mu5	GTGTCCGAGTTCCTTTCTGGGATCAAGCACTCTGGAAGCAGTCTGAGCATTGAGAAGGTG	2520
EH	CGAGACGAGATTGAGTGGAGAAGAAGGATGAGAGGAGTAAGAGACGAGATCCTTGCAGGA	2580
Mu5	CGAGACGAGATTGAGTGGAGAAGAAGGATGAGAGGAGTAAGAGACGAGATCCTTGCAGGA	2580
EH	CTCTGCTCCTCTGA-----	2595
Mu5	CTCTGCTCCTCTGAGTGAATAAACAGGGTTCTCTGTAAAAAAAAAAAAAAAAAAAAAAAAAAAA	2640
PRIMER 10 == ===		

Fig. 2.1 continued.

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EH  MKWIELMIGLMPMIEGRKMEGSSGTEFFPRRLDRTVVP SHYDLHVKVM DTGFCGNVGIRVD 60
Mu5  MKWIELMIGLMPMIEGRKMEGSSGTEFFPRRLDRTVVP SHYDLHVKVM DTGFCGNVGIRVD 60
Ec  MRWIKVMAGLLPMIGSKGADEKDSSQQ-RRLSRVVVPEHYDLHVKILDAGFCGSVGIRVM 59
    *:***::* **:* ** .: : . . .:: ***.*.***.*****::*:***.*****
EH  ISQPVDEIMMNAKELDVLSAGIEIDGRRIDGEVVMGDDEKEYEVVRIRFPST?TLEGYI 120
Mu5  ISQPVDEIMMNAKELDVLSAGIEIDGRRIDGEVVMGDDEKEYEVVRIRFPSTIPKGEYI 120
Ec  ISQDVSEIVLNAKELEIRDAGIVVEGARIPGRVVVGEAEKELEVVRIVFPSSLRAGPGYL 119
    ***.*.***::*****::.***::* **.*.***:: *** ***** **: * ** :
EH  VGEFCGDYNSGLVGFYKSGGESKVYSTHFEPTDARRVFPCTDQPDMAKATFSISIDAPSNL 180
Mu5  VGEFCGDYNSGLVGFYKSGGESKVYSTHFEPTDARRVFPCTDQPDMAKATFSISIDAPSNL 180
Ec  TMEFCGDYNSGLVGLYKSGGPKEVYSTHFEPTDARRAFPCTDQPDMAKATFKISIDAGSKF 179
    . *****.*****:*****.*****Primer 1*****.******: :
EH  VVLSNSPSVPSLRKEYGDRATYFTKTSKMSTYLVAFFVAGELEYIEDWSKSGVRLRVYGQ 240
Mu5  VVLSNSPSVPSLRKEYGDRATYFTKTSKMSTYLVAFFVAGELEYIEDWSKSGVRLRVYGQ 240
Ec  TVLANTQAIPSLREEYGDRKIYFEETCKMSTYLVAFFVAGELSYIEDWSKDGVRLRVYGD 239
    .***:::*****:***** *****:*.*****.*****.*****:
EH  GKEEASWGRYGLEVAKRCDYFEEYFGIEYEFPEKGMKIDMIGIPSFSGGAMENWGLIT 300
Mu5  GKEEASWGRYGLEVAKRCDYFEEYFGIEYEFPEKGMKIDMIGIPSFSGGAMENWGLIT 300
Ec  S-SEVEWGRYGLEVGKRCLEYFSEYFGVGYEFPRAGSAKIDMVGIPNFSSGAMENWGLIT 298
    .*.*****.***::**.*.***:****.* *****:***.*.*****
EH  FRKESLLYSDGKDSVEQKKNVAETVCHELGHMWFGNLVTMEWDDLWLNEGFATWISFKG 360
Mu5  FRKESLLYSDGKDSVEQKKNVAETVCHELGHMWFGNLVTMEWDDLWLNEGFATWISFKG 360
Ec  FRRESLLYVPGKSNVEDMKNVAGTVCHELGHMWFGNLVTMSWDDLWLNEGFATWVSFKG 358
    **:***** **.*.***:***** *****Primer 2*****:*****

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Fig.2.2. *E. hellem* human isolate (EH), *E. hellem* bird isolate (Mu5), and *E. cuniculi* (Ec) aminopeptidase predicted amino acid sequences. Gaps (-) represent spaces introduced into the aligned sequences by the CLUSTAL W multiple sequence alignment program to maximize the homology between the aligned sequences. Complete identity is indicated by asterisks (*) and similarity indicated by colons (:) below the alignment. Amino acids sequences containing the conserved HEXXH zinc binding motif and GAMEN motif are bolded. The start methionine is double underlined. At position 113 in the *E. hellem* human sequence one clone predicted isoleucine and one predicted threonine, as indicated.


```

EH  MSEIGKEYVDWDVEGEFVLWNVIRGMLLEDGLGKSHKIRMEVSDPGEIGEIFDAISYSKGA 420
Mu5  MSEIGKEYVDWDVEGEFVLWNVIRGMLLEDGLGKSHKIRMEVSDPGEIGEIFDAISYSKGA 420
Ec   MENIG-SVVSWDVWGEFVLWNVVRGMVDDGLGKSHQIRMNVTDPGEIGEIFDSISYCKGA 417
      *.:** . *.*** *****:***::*****:***:*.*****:***.***
EH  SIYRMVEKYVGESVFMMSGIRRYIKEHMYGNGNGMSLWKAIGESYKDISSMVEGWISQAG 480
Mu5  SIIRMVEKYVGESVFMMSGIRRYIKEHMYGNGNGMSLWKAIGESYKDISSMVEGWISQAG 480
Ec   SVIRMIERYVGESVFMMLGIRRYIKEHMYGNGNAMS L WKAIGE EYGEDISEMVEGWISQAG 477
      *:***:*.***** *****.*****. **:***.*****
EH  YPVISVSESGENLVLSQRRYSMLGKSDDSMWIVPVVITWGSGENESIEVGGEREVVIPRRS 540
Mu5  YPVISVSESGENLVLSQRRYSMLGKSDDSMWIVPVVITWGSGENESIEVGGEREVVIPRRS 540
Ec   YPVVSVQDCGSSLVLSQSRYSM LGKSDDSLWTIPVVVSWEGKGQERIELRGRET TVRKRS 537
      ***:*.:.*. ***** *****: * :***::* . :* ** : **.: :**
EH  DVYKVNSSYAGFYRVLYEADALRRLEERLEEMGMDRVNMVEDVFG LGFGLYGGIGERLR 600
Mu5  DVYKVNSSYAGFYRVLYEADALRRLEERLEEMGMDRVNMVEDVFG LGFGLYGGIGERLR 600
Ec   SVYKVNAEYGGFYRVLYDSAGLSGLESRIDSLSVVD RVNVIEDVFG LGFGLYGGLEHGLR 597
      .*****:*. *****: : . * ** .*: : . : . :*****:*****: . **
EH  KIGEYYS DGYCVARSTVEKLMRIRSVFYDDAEIVSAVDKRVLG LIEERIESIDTSVCETS 660
Mu5  KIGEYYS DGYCVARSTVEKLMRIRSVFYDDAEIVSAVDKRVLR LIEERIESIDTSVCETS 660
Ec   RISEYYS DSYHVARSGIEKLLRLRSVFYDDAEIVSLIDKKVRKMILPCVGRIDVFDIGTS 657
      :*.*****.* **** :***:*.*****:***:* :* : ** . **
EH  VERISMNKYLLSVGIEVGSASAMRKVSELWRRHVEEGAELGELRWIVYKGVVDDNLEHLI 720
Mu5  VERISMNKYLLSVGIEVGSASAMRKVSELWRRHVEEGAELGELRWIVYKGVVDDNLEHLI 720
Ec   VESVSMNKYVLSVGVEVGIREAVEKVQELWRRHVEAGEELGELRWIVYKAVVDENLG YMM 717
      ** :*****:*****:*** .*: .*. ***** * *****.***:*** :::
EH  GEYKSGRRPGARWEVINGFSGIKRKESFAEVVKRLCEFSVEDIGVVIAIICRSGNFRDEM 780
Mu5  GEYKSGRRPGTRWEVISGFSGIKRKESFAEVVKRLCEFSVEDIGVVIAIICRSGNFRDEM 780
Ec   DKHKYGDTPGMRREVMNGFSGIKREENFLDVVGNLSQFSVEDIGVVIGSISRGGAFRDAM 777
      .::* * * * * :.*****:*. * :** .*. :*****. *. * * * *
EH  VEYVVSHREELYSMVQKNVMLYNTIVMSLRHVSSDPMVEKVSEFLSGIKHSGSSLSIEKV 840
Mu5  VEYVVSHREELYSMVQKNVMLYNTIVMSLRHVSSDPMVEKVSEFLSGIKHSGSSLSIEKV 840
Ec   VEYVVSHGEELYLMVHKNAMLYNMIIMSLRHVSGDLIVEKVERFLSGIKHSGSNLSIEKV 837
      ***** ***** **:***.***** *:*****. * :*****.*****.*****

```

Fig. 2.2 continued.

```

EH  RDEIEWRRRMRGVRDEILAGLCSE----- 865
Mu5 RDEIEWRRRMRGVRDEILAGLCSEINRVLCKKKKKKKKKKVLCVVTTACPIVSRIRAN 900
Ec  RNEIQWRRRMRGIREEVLRLPEAEK----- 864
    *:***:*****:***:*** ** ..

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Fig. 2.2 continued.

Aminopeptidase Gene Sequence Alignments

E. hellem (bird) cDNA and gDNA

Three differences were observed in the aligned aminopeptidase gene nucleotide sequences obtained from the *E. hellem* bird isolate cDNA and gDNA. At position 1485, an A in the cDNA sequence was replaced with G in the gDNA sequence, resulting in no change in the predicted amino acid sequences. At position 1927, 2 different cDNA clones had an A or a G resulting in codons for arginine or glycine, respectively. In the gDNA sequence, a G was in position 1927, predicting glycine. At position 2248, a G was found in the cDNA sequence and an A in the gDNA sequence, resulting in no change in the predicted amino acid sequence.

E. hellem (human) cDNA and gDNA

Six differences were observed in the aligned aminopeptidase gene nucleotide sequences obtained from the *E. hellem* human isolate cDNA and gDNA. At position 338, 2 different cDNA clones had a C or a T resulting in codons for threonine or isoleucine, respectively. In the gDNA sequence, a T was in position 338 predicting isoleucine. At position 375, a C in the cDNA sequence was replaced with T in the gDNA sequence, resulting in no change in the predicted amino acid sequences. At position 1038, 2 different cDNA clones had an A or a G the gDNA sequence, resulting in no change in the predicted amino acid sequences. At position 1267, a G in the cDNA sequence predicts a valine and was replaced with an A in the gDNA sequence, which predicts isoleucine. At position 1539, an A in the cDNA sequence was

replaced with G in the gDNA sequence, resulting in no change in the predicted amino acid sequences. At position 1617, a G in the cDNA sequence was replaced with A in the gDNA sequence, resulting in no change in the predicted amino acid sequences.

E. hellem (bird) and *E. hellem* (human) cDNA nucleotide sequence comparison

The cDNA aminopeptidase nucleotide sequences obtained from *E. hellem* bird and human isolates were aligned and compared. The sequences were >99 % identical. Differences were observed at 14 different positions (231, 338, 1254, 1267, 1617, 1927, 1095, 1485, 1539, 2191, 2202, 2210, 2247 and 2466) resulting in differences in the predicted amino acids at 4 of these positions (338, 1267, 2191, and 2210) (Table 2.2 and 2.3).

At positions 231, 1254, 1617, 1927 and 2202, a G in the human cDNA sequence was replaced with an A in the bird isolate cDNA sequence. At positions 1095, 1485, 1539, and 2247, an A in the human cDNA sequence was replaced with a G in the bird isolate cDNA sequence. At position 2466, a T in the human cDNA sequence was replaced with a C in the bird isolate cDNA sequence. These substitutions resulted in no changes in the predicted amino acid sequences (Table 2.2 and Fig. 2.1).

At position 338, 2 different human isolate cDNA clones had a C or a T resulting in codons for threonine or isoleucine, respectively. At position 338, the bird isolate cDNA clone had a T and predicted threonine. At position 1267, a G in the human cDNA sequence was replaced with an A in the bird isolate cDNA sequence, predicting valine and isoleucine, respectively. At position 2191, a G in the human

cDNA sequence was replaced with an A in the bird isolate cDNA sequence, predicting alanine and threonine, respectively. At position 2210, an A in the human cDNA sequence was replaced with a G in the bird isolate cDNA sequence, predicting arginine and serine, respectively (Table 2.3 and Fig. 2.1 and 2.2).

To summarize, there were 4 differences among the *E. hellem* aminopeptidase nucleotide sequences that resulted in changes in the predicted amino acid sequences. Due to a C substituted for a T at position 338, threonine was encoded for by 1 human isolate cDNA clone, but the sequences from a second human isolate cDNA clone and gDNA, and the bird isolate cDNA and gDNA all coded for isoleucine with a T at this position. Next, at position 1267, the *E. hellem* human isolate cDNA sequence contains a G predicting valine and the gDNA sequence contains an A and predicts isoleucine, while the cDNA and gDNA sequences of the bird isolate contain an A. At position 2191, the *E. hellem* human isolate cDNA and gDNA sequences have a G at this position and predict an alanine. The bird cDNA and gDNA sequences have an A and predict a threonine. Lastly, at position 2210, the *E. hellem* human isolate cDNA and gDNA sequences contain an A and predict asparagine while the bird cDNA and gDNA sequences have a G and predict a serine (Table 2.3, Fig. 2.1 and 2.2).

Table 2.2. Silent nucleotide differences in the *E. hellem* aminopeptidase gene sequences.

Isolate	Sequence	Position	Nucelotide	Isolate	Sequence	Position	Nucelotide
human	cDNA	231	G	human	cDNA	1617	G
bird	cDNA	231	A	bird	cDNA	1617	A
human	cDNA	1095	A	human	cDNA	1927	G
bird	cDNA	1095	G	bird	cDNA	1927	A
human	cDNA	1254	G	human	cDNA	2202	G
bird	cDNA	1254	A	bird	cDNA	2202	A
human	cDNA	1485	A	human	cDNA	2247	A
bird	cDNA	1485	G	bird	cDNA	2247	G
human	cDNA	1539	A	human	cDNA	2466	T
bird	cDNA	1539	G	bird	cDNA	2466	C

Table 2.3. Nucleotide differences in the *E. hellem* aminopeptidase gene sequences resulting in a change in the predicted amino acid.

Isolate	Sequence	Position	Nucelotide	Position	Predicted amino acid
human	cDNA	338	T/C	113	Isoleucine/Threonine
bird	cDNA	338	A	113	Isoleucine
human	cDNA	1267	G	422	Valine
bird	cDNA	1267	A	422	Isoleucine
human	cDNA	2191	G	731	Alanine
bird	cDNA	2191	A	731	Threonine
human	cDNA	2210	A	737	Asparagine
bird	cDNA	2210	G	737	Serine

E. cuniculi (rabbit) and E. cuniculi GenBank CAA06646

The *E. cuniculi* aminopeptidase sequence obtained in this study was 100 % identical to the *E. cuniculi* zinc metalloprotease sequence in the GenBank database (GenBank accession no. CAA06646). The sequence was also aligned and compared with an *E. cuniculi* glutamyl aminopeptidase (GenBank accession no. NP_597136) and only 3 substitutions in the predicted amino acid sequences were observed, resulting in an identity value of >99 %.

E. hellem human and bird and E. cuniculi predicted amino acid sequences

The aligned predicted amino acid sequences of *E. hellem* human and bird aminopeptidases shared >99% identity. The *E. hellem* human and bird predicted aminopeptidase sequences shared >69% identity with the *E. cuniculi* aminopeptidase predicted amino acid sequence.

Protein Motif Blast Search and Protein Mass Prediction

The predicted mass of the aminopeptidase sequences of each isolate was approximately 97 kDa. Similarity searches based on conserved protein motifs identified HEXXH zinc binding region and GAMEN motifs. These motifs classify the enzymes as zinc metalloproteases of the M1 family of aminopeptidases.

Protein mass predictions of the *E. cuniculi* zinc metalloprotease, glutamyl and leucine aminopeptidase sequences (GenBank accession nos. CAA06646, NP_597136, NP_586627) were approximately 97, 97, and 70 kDa, respectively. Similarity searches based on conserved protein motifs identified HEXXH zinc binding

region and GAMEN motifs in the zinc metalloprotease and glutamyl aminopeptidase sequences (GenBank accession nos. CAA06646 and NP_597136), also classifying these aminopeptidase as a zinc metalloprotease of the M1 family of aminopeptidases. Similarity searches of the leucine aminopeptidase sequence (GenBank accession no. NP_586627) classify this enzyme under the M17 family of aminopeptidases.

Discussion

The *E. hellem* and *E. cuniculi* aminopeptidases reported here show sequence homology with other aminopeptidases classified under the M1 family in the MA clan of aminopeptidases. The sequences contain the conserved HEXXH + E zinc binding and GAMEN motifs (Fig. 2.2), and thereby indicate that the sequences code for metalloproteases with at least one zinc atom required for catalytic activity (Rawlings & Barret, 1995). These conserved motifs showed sequence homology with aminopeptidases of other organisms such as *Caenorhabditis elegans*, *Haemonchus contortus*, and *Aspergillus niger* (GenBank accession nos. NP_5501220.1; CAB57357.1; CAC38353.1).

Members of the M1 family of aminopeptidases include membrane bound and cytosolic aminopeptidases, and many members are heavily glycosylated (Rawlings & Barrett, 1995). Members of the M1 family include *E. coli* aminopeptidase N; alanine/arginine aminopeptidase from *Saccharomyces cerevisiae*; leucine aminopeptidase (lap) from *Lactococcus lactis*; lysine aminopeptidase of *A. niger*; arginine aminopeptidase of *C. parvum* and zinc metallopeptidase of *P. falciparum* (Taylor, 1993; Okhuysen *et al.* 1994; Rawlings & Barrett, 1995; Florent *et al.* 1998;

Basten, Visser & Schaap, 2001; Allary, Schrevel & Florent, 2002). Interestingly, although aminopeptidases may show preferential cleavage of certain substrates, many members of the M1 family demonstrate the ability to cleave a variety of substrates. Examples are the aminopeptidase of *P. falciparum*, which efficiently hydrolyzes lysine, alanine, arginine, and leucine (Allary *et al.* 2002); the arginine aminopeptidase of *C. parvum* which can hydrolyze alanine, phenylalanine, glycine, and leucine as well (Okhuysen *et al.* 1994); and the lysine aminopeptidase of *A. niger* which also cleaves arginine, methionine, alanine, leucine, and phenylalanine (Basten *et al.* 2001).

The obtained open reading frames of the *E. hellem* bird isolate, *E. hellem* human and *E. cuniculi* produced predicted amino acid sequences of 865, 864, and 863 residues, respectively. The *E. hellem* human and *E. cuniculi* aminopeptidase genes were acquired by PCR, thus the ends are incomplete compared to the sequence produced by RACE for *E. hellem* bird. Also *E. cuniculi* has a three base deletion within the gene resulting in a shorter amino acid sequence. No introns were found in the genomic DNA sequences of either the *E. cuniculi* or *E. hellem* aminopeptidases in this study. This is consistent with previous data, which has found only a single intron in the entire genome from *E. cuniculi*, located at the beginning of the large subunit ribosomal protein of *E. cuniculi* (Biderre *et al.* 1998).

The *E. hellem* isolates shared >99 % identity of both nucleotide and predicted amino acid sequences. A few differences were observed at the nucleotide level between cDNA and gDNA sequences of the same *E. hellem* isolate and between the cDNA sequences of the two *E. hellem* isolates. Most of these differences did not

change the predicted amino acid sequence and were therefore likely due to codon usage. Four of these differences resulted in a change in predicted amino acid sequences, but they did not occur at regions critical to enzyme activity. In addition, the changes that were observed involved neutral substitutions, and are therefore probably of no consequence. A high fidelity polymerase with proofreading activity was used for all PCR reactions therefore error due to PCR amplification is unlikely.

The *E. hellem* sequences shared nearly 70 % identity with that of *E. cuniculi* at both the nucleotide and predicted amino acid sequence levels. The predicted amino acid sequence of *E. cuniculi* was identical to the *E. cuniculi* zinc metalloprotease (GenBank accession no. CAA06646), and was >99 % identical to that of *E. cuniculi* glutamyl aminopeptidase sequence (GenBank accession no. NP_597136).

The *E. hellem* and *E. cuniculi* sequences correspond in length with those of *E. cuniculi* zinc metalloprotease (GenBank accession no. CAA06646) and *E. cuniculi* glutamyl aminopeptidase (GenBank accession no. NP_597136), which are 864 amino acids in length. These sequences are much longer than those of *E. cuniculi* leucine and methionine (GenBank accession nos. NP_586294 and NP_586190, respectively) aminopeptidases, which are 486 and 358 amino acids in length, respectively. Thus, the aminopeptidase genes sequenced in this study are most likely homologous to the *E. cuniculi* glutamyl aminopeptidase described in GenBank (accession no. NP_597136).

The *E. hellem* and *E. cuniculi* aminopeptidase gene sequences obtained in this study all have predicted molecular masses of approximately 97 kDa. The *E. cuniculi*

glutamyl aminopeptidase sequence (Genbank no. NP_597136) also has an estimated predicted mass of 97 kDa and the gene sequence is 99% identical to that obtained for *E. cuniculi* in this study. The *E. cuniculi* aminopeptidase sequence in this study is identical to the GenBank sequence CAA06646, which is also 99% identical to the *E. cuniculi* glutamyl aminopeptidase (GenBank Accession No. NP_597136) and also has a predicted molecular mass of 97 kDa. Although these 2 aminopeptidase genes (CAA06646 and NP_597136) are located on 2 different chromosomes, 1 and 8 respectively, the high sequence identity suggests that they are homologous with similar functions. It is therefore it is probable that the differences observed between these 2 sequences may be attributed to the fact that they represent dual copies located on different chromosomes.

Based on motif and predicted mass similarities among the *E. hellem* aminopeptidase obtained in this study and the *E. cuniculi* aminopeptidases (GenBank CAA06646 and NP_597136), it is likely that these sequences code for homologous enzymes. Therefore, it is likely that the *E. hellem* aminopeptidases are also glutamyl aminopeptidases. Clearly, additional work is needed to confirm the substrate specificity of the *E. hellem* enzymes.

There are currently 3 types of aminopeptidases, glutamyl, leucine and methionine (GenBank accession nos. NP_597136, NP_586294 and NP_586190, respectively), which have been identified in the genome of *E. cuniculi* (Katinka *et al.* 2001). These aminopeptidases are classified under 3 different families, M1, M17, and M24, respectively, based on conserved motifs identified in each sequence. Previous

studies by Millership *et al.* (2002a, 2002b) have identified aminopeptidase activity from *E. intestinalis*, *E. cuniculi* and *E. hellem*. The enzymatic activity was inhibited by bestatin and nitrobestatin *in vitro* and showed preferential cleavage of the substrate leucine. Activity against arginine and to a lesser extent L- methionine, but not Z- methionine, was also shown (Millership *et al.* 2002a; 2002b). The estimated molecular masses of the aminopeptidases of *E. intestinalis*, *E. cuniculi* and *E. hellem* were 70, 74 and 72, respectively, determined by substrate gel electrophoresis (Millership *et al.* 2002a; 2002b). It appears that these aminopeptidases from these 3 species are homologous enzymes since they share substrate specificity and similar molecular masses.

The predicted molecular masses of the *E. cuniculi* glutamyl, leucine, and methionine aminopeptidases in the GenBank database were 97, 50, and 40 kDa, respectively. The aminopeptidases described by Millership *et al.* (2002a, 2002b) that preferentially cleaved leucine may or may not be homologous to the *E. cuniculi* leucine aminopeptidase (GenBank no. NP_586294). The aminopeptidases obtained by Millership *et al.* have molecular masses ranging from 70 to 74 kDa based on substrate gel electrophoresis, whereas the leucine aminopeptidase has a molecular mass of 50 kDa predicted from the amino acid sequence. This leucine aminopeptidase possesses 3 potential glycosylation sites. Glycosylation adds mass by the addition of carbohydrate chains, therefore, the actual molecular mass could be considerably greater than the predicted value if the enzyme is glycosylated,. Thus, it is possible that the aminopeptidase reported by Millership *et al.* is homologous to the reported leucine

aminopeptidase (GenBank no. NP_586294). Based on the strong substrate preference for leucine and the limited cleavage of methionine reported by Millership *et al.* for the *Encephalitozoon* aminopeptidase, it is not likely that this enzyme is homologous to the *E. cuniculi* methionine aminopeptidase in the GenBank database (GenBank accession no. NP_586190).

The predicted molecular mass of an identified zinc metalloprotease of *Plasmodium falciparum* is 122 kDa; the precursor protein is 120 kDa based on western blot analysis (Florent *et al.* 1998; Allary *et al.* 2002). The precursor protein is further processed into two enzymatically active subunits, p96 and p68 (Allary *et al.* 2002). If the 97 kDa glutamyl aminopeptidase of *E. cuniculi* (GenBank accession no. NP_597136) undergoes similar processing resulting in an active enzyme with a lower molecular mass, it is possible that the 70 kDa aminopeptidase obtained by Millership *et al.* could correspond to a similarly processed enzyme. Since glutamate was not a substrate tested by Millership *et al.* (2002a, 2002b) the relationship between these 2 enzymes is speculative at best.

Genomes of microsporidia are characteristically very small and have very low copy number (Weiss *et al.* 1999). The high degree of interspecies identity among the *E. hellem* and *E. cuniculi* aminopeptidases and the dual copies of the homologous genes confirmed in the *E. cuniculi* genome (Weiss *et al.* 1999) suggest that this enzyme may play a critical role in the life cycle of these organisms. This enzyme would thus be an ideal candidate for further evaluation as a tool for drug design and potential therapy.

CHAPTER III

IN VITRO GROWTH COMPARISONS

Introduction

Microsporidia are obligate intracellular spore forming parasites found worldwide with a wide host distribution. Diseases caused by microsporidia have been documented in invertebrates, birds, mammals, and humans. In addition, there is increasing evidence that some species may be zoonotic (Deplazes *et al.* 2000).

Microsporidial infections with members of the genus *Encephalitozoon* have been documented in a variety of mammals and birds, as well as in human beings. *Encephalitozoon cuniculi* was first documented in rabbits (Wright & Craighead, 1922) and has since been described in a range of mammals, including humans and domestic dogs and cats (Deplazes *et al.* 2000). In addition, molecular analysis of sequences of the intergenic transcribed spacer (ITS) region of different *E. cuniculi* isolates have classified them into 3 genotypes, 2 of which have been obtained from both human and animal hosts (Deplazes *et al.* 1996; Didier *et al.* 1996; Mathis *et al.* 1997; Snowden *et al.* 1999). *Encephalitozoon hellem* was first identified from human AIDS patients in 1991 (Didier *et al.* 1991a) and was later described in a number of different bird species (Carlisle *et al.* 2002).

Molecular analyses and experimental infections of *E. hellem* human and bird isolates reveal shared characteristics between isolates. Sequence comparisons of ribosomal RNA genes from avian and human isolates show nearly 100 % identity among isolates sequenced, suggesting conspecificity. In addition, molecular

characterization of ITS regions of avian isolates classifies these isolates as genotype I, which is also the most frequently described genotype of human *E. hellem* isolates (Suter *et al.* 1998; Pulparampil *et al.* 1998; Snowden & Logan, 1999); Snowden *et al.* 2000; Snowden *et al.* 2001). Experimental infections of budgerigar chicks (*Melopsittacus undulates*) with avian and human *E. hellem* isolates showed no clinical or parasitological differences between the isolates (Snowden, personal communications). A recent epidemiological survey of lovebirds identified *E. hellem* spores in 25 % of bird samples (Barton, Phalen & Snowden, 2003).

In vivo studies, molecular analysis, and epidemiologic data support the probability of microsporidiosis being zoonotic. It is also probable that birds may be natural hosts of *E. hellem* and, therefore, pet birds may pose a health risk for immune compromised owners. However, further evidence is needed to prove this hypothesis.

In this study, bird and human isolates of *E. hellem* and one rabbit isolate of *E. cuniculi* were evaluated under simulated avian and mammalian *in vitro* conditions. The preferred growth conditions of these parasites were evaluated based on growth curves derived from the numbers of spores produced by each isolate.

Materials & Methods

Encephalitozoon isolates

The bird isolate of *E. hellem* was isolated from droppings of a lovebird (*Agapornis roseicollis*) (Snowden *et al.* 2000). The human *E. hellem* isolate was from an AIDS patient suffering from keratoconjunctivitis (Didier, 1991). The *E. cuniculi* isolate was originally obtained from a rabbit (Shadduck, 1969). All isolates were kindly provided as cryopreserved spore pellets by Dr. Karen Snowden.

Spore Collection

Spores were collected as described in Chapter II for inoculation of plated cells.

Mammalian Model (Experiment 1)

The RK-13 rabbit kidney cell line (ATCC CCL-37) was grown in 75 cm² flasks to confluency in Dulbecco's modification of Eagle's minimal essential medium (DMEM, Sigma, St. Louis, MO) supplemented with L-glutamine (2mM) (Gibco BRL, Grand Island, NY), 5 % heat inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO) and antibiotic/antimycotic (Gibco BRL, Grand Island, NY) at 37 °C and a humidified 5 % CO₂ in air atmosphere. The cells were routinely maintained by discarding old media and feeding cells with 24 ml fresh media twice weekly. Once cells reached confluency they were detached by trypsinization. The medium was removed and the cell layers were washed with 10 ml 0.15 M phosphate-buffered saline

(PBS) (Sigma, St. Louis, MO). The cells were detached from the flask by incubating with 5 ml trypsin (0.05% trypsin; 0.53 mM EDTA) for 5-10 min. Cells were resuspended with the addition of 10 ml medium with 5 % serum to inactivate the trypsin. One ml of the cell dilution was used to continue growth of the cells until ready for use in the experiment after approximately 8 passages.

Cells were detached and harvested for the experiment by trypsinization as described above. Percent viability was calculated using a 1:1 dilution of a 100 µl cell suspension sample and Trypan Blue stain. Nonviable and viable cells were counted with a hemocytometer. RK-13 cells at a concentration of 3.5×10^5 viable cells/well were plated in 24 well plates in 1.5 ml cell culture medium. After 48 h the cells reached confluency and were inoculated with 1.5×10^6 spores/well. Spores from the *E. hellem* human and bird isolates and *E. cuniculi* were collected as described in Chapter II, resuspended in cell culture medium and quantified using a hemocytometer before inoculating plates. Three separate 24 well plates were maintained for each isolate. Plates were incubated at 37 °C and a humidified 5 % CO₂ in air atmosphere during the experiment. Mammalian Experiment 1 was duplicated.

Avian Model (Experiments 2 and 3)

Experiment 2

A chicken fibroblast cell line (chicken embryonic fibroblast, ATCC CRL-12203) was grown in 75 cm² flasks to confluency in DMEM with 2 mM L-glutamine containing 5 % heat inactivated chicken serum (CS) and antibiotic/antimycotic at 40

°C and a humidified 5 % CO₂ in air atmosphere. The cells were routinely maintained by removing old media and feeding cells with 24 ml fresh media twice weekly. Once cells reached confluency they were detached by trypsinization. Trypsinized cells were diluted in 20 ml medium and 0.5 ml of the 1:25 cell dilution was used to continue growth of the cells until ready for use in the experiment after approximately 4 passages. The cells were trypsinized and viable cells quantified as described above, then plated at a concentration of 1.0×10^4 viable chicken cells/well in 24 well plates. Plates were inoculated the following day with 5.0×10^4 spores/well with spores from the *E. hellem* human and bird isolates and *E. cuniculi* collected and quantified as described above. Three separate 24 well plates were maintained for each isolate. Plates were incubated at 40 °C and a humidified 5 % CO₂ in air atmosphere during the experiment.

Experiment 3

Experiment 3 was conducted as Experiment 2, except that plates were inoculated with 1.5×10^6 spores/ well. Experiment 3 was duplicated.

Growth Curve Determination

The spores and cells in triplicate wells were harvested every 3 days for the mammalian model Experiment 1 and the avian model Experiment 2, but every 2 days for the avian model Experiment 3, beginning at day 2 after inoculation. A total of 8 time point values were obtained for each isolate for each experiment.

At harvests plates were first centrifuged at 800 *g* for 12 min to minimize the loss of extra-cellular spores. Next, 1 ml of medium was removed from non-harvest wells and replaced with fresh medium. All media was then removed from the harvest wells, and the wells were washed with PBS. The cells were trypsinized with 1 ml trypsin, transferred to a 15 ml tube, and washed in 10 ml sterile tap H₂O to lyse host cells. Cells and spores were recovered by centrifugation for 15 min at 800 *g*. The supernatant was discarded and the pellets were resuspended in 100 µl 1 % sodium dodecyl sulphate (SDS) and allowed to stand for 5 min. The suspensions were next washed in H₂O and centrifuged at 800 *g* for 15 min. The supernatant was discarded and pellets were resuspended in 1 ml 1 % formalin. The total number of spores/well was quantified using a hemocytometer with a minimum of 2 counts per sample. The average of each count for each well was determined and the time point value then calculated as the average of the 2 counts of triplicate wells (6 values) for the duplicate experiments. Experiment 2 was not duplicated, so each time point value was derived from a single set of 3 wells. The obtained time point values were graphed to yield a growth curve using Graph Pad PRIZM 3.0 and the data was analyzed using Statistical Analysis System (SAS) software (SAS Institute 1996).

Statistical Analysis

Isolate Comparisons within Experimental Model

Statistical methods were used to analyze differences between the growth of isolates under the same experimental conditions. A mixed model with repeated

measures and multivariate linear regression was used for analyses. Growth data (number of spores/well; 3 samples) were transformed by $\text{Log}_{10}(X)$ before analysis using SAS to test for significant differences (P value ≤ 0.05).

Isolate Comparisons between Experimental Models

In addition, statistical methods also were used to compare the same isolate under different experimental conditions at selected days post inoculation. Data were analyzed by multivariate linear regression, and data (number of spores/well; 3 samples) were $\text{Log}_{10}(X)$ transformed before analyses using SAS software to test for significant differences (P value ≤ 0.05). Days 2, 8, and 14 post inoculation (PI) were selected for isolate growth comparison from the different experiments because parasite harvests coincided at these days.

Results

Isolate Comparisons within Experimental Model

Mammalian Model (Experiment 1)

A total of 1.5×10^6 spores/well for each isolate was inoculated into 24 well plates with confluent RK cells. Plates containing each isolate were maintained at 37 °C for a duration of 24 days. Comparative growth curves are illustrated in Fig. 3.1 and the spore averages calculated for each y-value are located in Appendix I. No statistical differences were observed between duplicate experiments. Overall, *E. cuniculi* grew significantly better than the 2 *E. hellem* isolates ($P \leq 0.05$, Appendix

II). The log phase of *E. cuniculi* begins at day 2 PI and continues until day 11 PI, at which time 3.5×10^7 spores were produced. Days 14-24 represented a stationary phase with approximately the same spore count.

The *E. hellem* isolates grew at similar rates. The average numbers of spores were significantly different ($P=0.0087$) for the *E. hellem* isolates at day 2 PI, with the bird isolate growth significantly lower than that of the human isolate ($P=0.0087$). However, this represents a point in the lag phase of the microsporidial growth and did not appear to influence later growth since at time point 5 (day 14 PI), the bird isolate produced significantly more spores than the human isolate ($P \leq 0.0027$). The number of *E. hellem* isolate spores steadily increased from day 5 to 20 PI, to a maximum of 2.1×10^6 and 3.5×10^6 for the bird and human isolates, respectively. This was significantly lower compared to a maximum of 3.5×10^7 for *E. cuniculi* on day 11. The number of *E. hellem* spores produced remained stationary between day 20 and day 23 PI (Fig. 3.1).

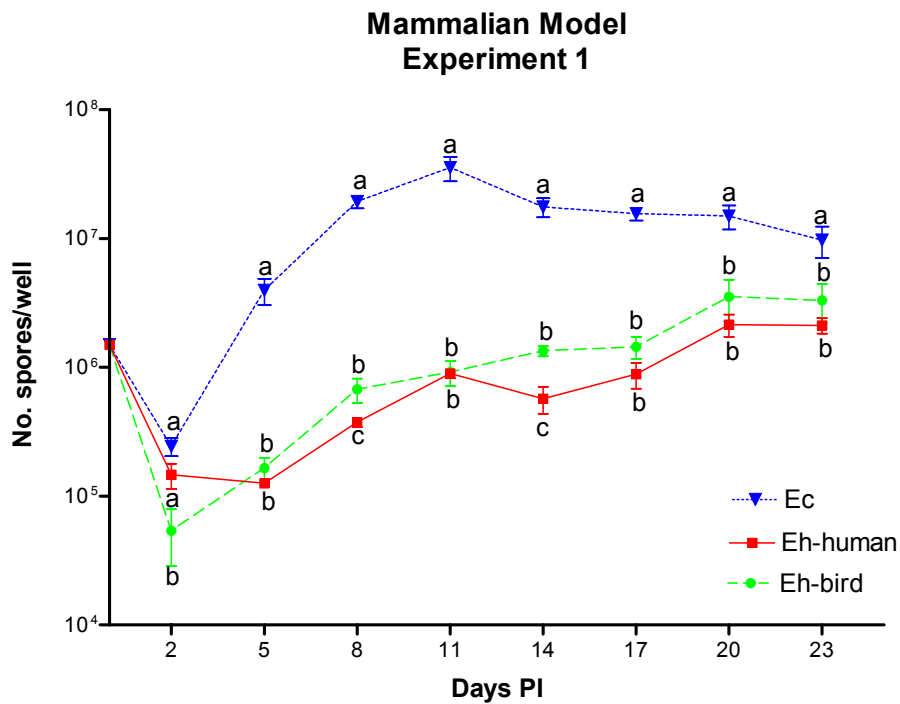


Fig. 3.1. *In vitro* growth curves of microsporidia isolates under Experiment 1 mammalian conditions. The average number of spores/well for duplicate experiments at each time point is plotted for *E. cuniculi* (Ec ▼), *E. hellem* human (Eh-human ■), and *E. hellem* bird (Eh-bird ●). Rabbit kidney cells were inoculated with 1.5×10^6 spores/well and maintained at 37 °C for a period of 24 days. Lower case letters a,b and c represent significant differences between isolates ($P \leq 0.05$).

Avian Model

Experiment 2

A total of 5.0×10^4 spores/well for each isolate was inoculated into 24 well plates seeded with 1.0×10^4 chicken embryonic fibroblast cells. The plates were maintained at 40 °C for 24 days. The results for Experiment 2 are graphed below (Fig. 3.2) and the spore averages calculated for each y-value are located in Appendix I. Overall, the *E. hellem* bird isolate grew significantly better than both the *E. hellem* human isolate and the isolate of *E. cuniculi* ($P \leq 0.05$). The numbers of spores steadily increased for the *E. hellem* bird isolate and *E. cuniculi* from days 2 -20 PI. At day 20 PI the *E. hellem* bird isolate reached a maximum of 3.0×10^6 spores compared to 7.3×10^5 for *E. cuniculi*. Although a decrease in *E. hellem* bird spore number was observed between days 20 and 23 PI, while the number of spores remained stationary for the *E. cuniculi* isolate between these days, the *E. cuniculi* spore counts were significantly lower than the *E. hellem* bird counts on these days ($P < 0.0001$, 0.0044). The maximum value for *E. hellem* human was 5.1×10^5 on day 17, which was significantly lower than the corresponding value for *E. hellem* bird ($P < 0.0001$), but not significantly different from *E. cuniculi* ($P = 0.9912$). No statistical differences were observed between *E. cuniculi* and the *E. hellem* human isolates at days 14-23 PI (Fig. 3.2).

A significant lag in growth was observed for the *E. hellem* human isolate between days 2-5 PI, which may have resulted in lower spore counts seen for *E. hellem* human for the duration of the experiment, although the curve steadily

increased between days 5-17 PI. Between days 17 and 20 PI there was a slight drop in the *E. hellem* human spore count, and the counts then remained stationary between days 14, 17 and 23 PI.

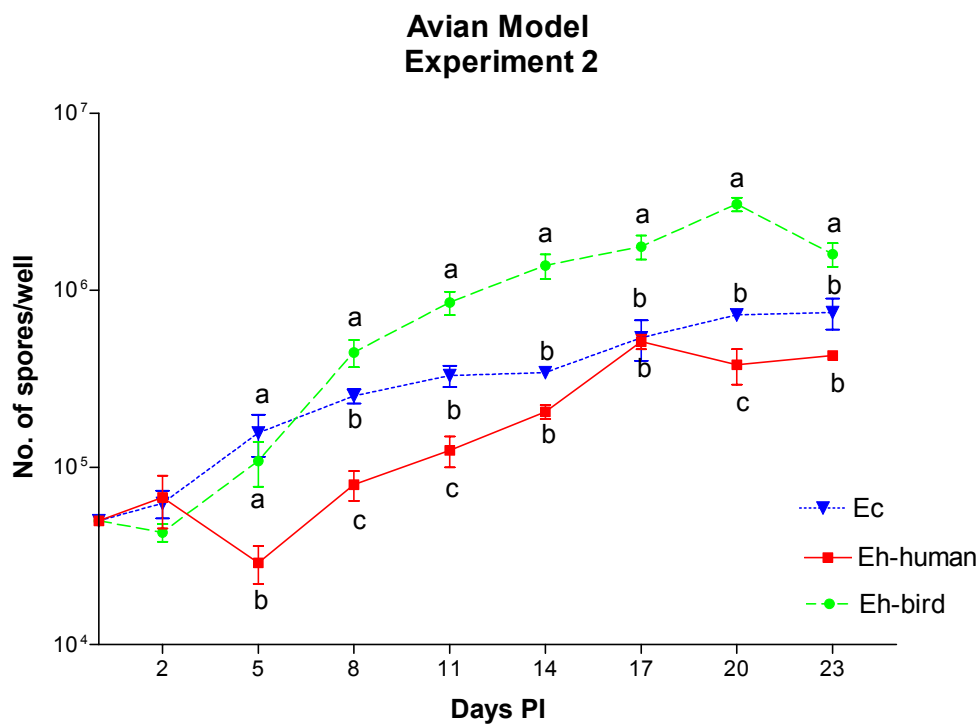


Fig. 3.2. *In vitro* growth curves of microsporidia isolates under Experiment 2 avian conditions. The average number of spores/well at each time point is plotted for *E. cuniculi* (Ec ▼), *E. hellem* human (Eh-human ■), and *E. hellem* bird (Eh-bird ●). Chicken cells were inoculated with 5.0×10^4 spores/well and maintained at 40°C for a period of 24 days. Lower case letters a, b and c represent significant differences between isolates ($P \leq 0.05$).

Experiment 3

A total of 1.5×10^6 spores/well for each isolate was inoculated into 24 well plates seeded with 1.0×10^4 chicken embryonic fibroblast cells. Plates containing each isolate were maintained at 40 °C, and the duration of the experiment was 16 days. Significant differences were not observed between the isolates for the duration of the experiment ($P \leq 0.05$, Appendix II). Comparative growth curves of Experiment 3 conducted in duplicate are graphed below (Fig. 3.3) and the spore averages calculated for each y-value are located in Appendix I.

An increase in the number of spores was observed for all three isolates between days 2 and 4 PI, which subsequently decreased between days 4 and 6 PI. Overall the spore numbers of all 3 isolates began to increase between days 6–16 PI. However, a drop in parasite numbers was observed for the bird isolate of *E. hellem* at days 8 and 12 PI, but was statistically insignificant ($P > 0.05$, Appendix II). Although the numbers increased between days 6–16 PI, a typical growth curve including lag, logarithmic, and stationary phases was not observed.

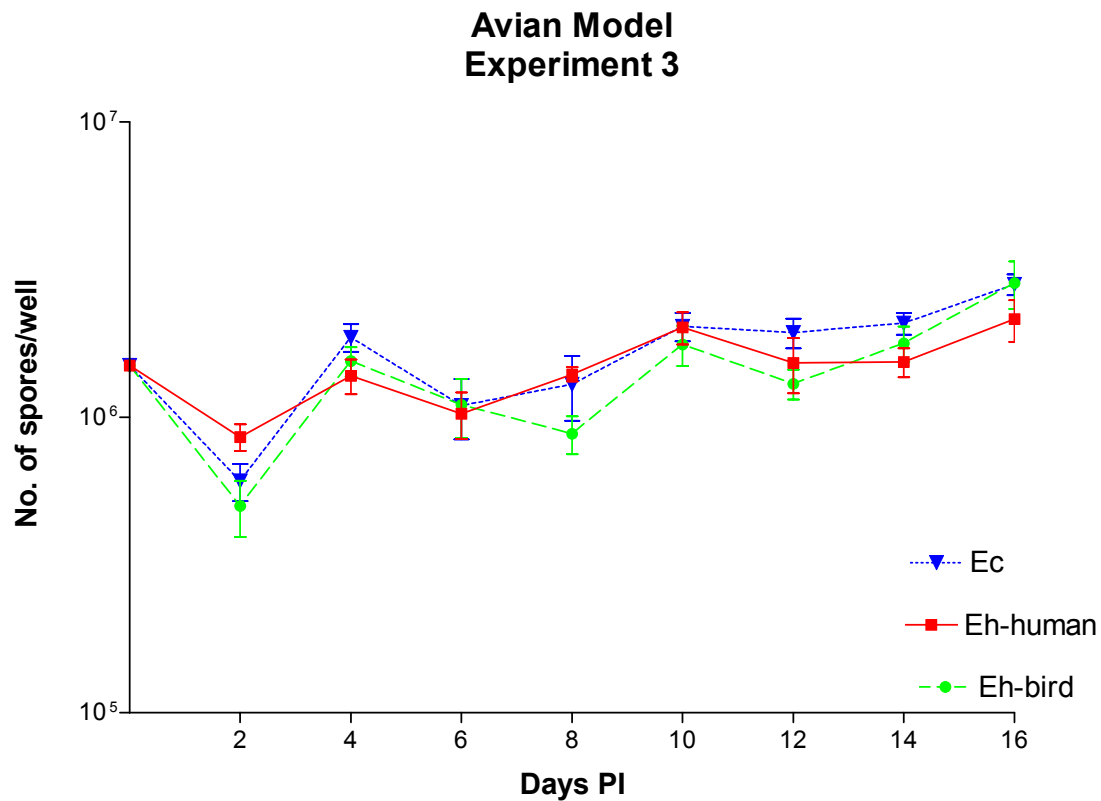


Fig. 3.3. *In vitro* growth curves of microsporidia isolates under Experiment 3 avian conditions. The average number of spores/well at each time point is plotted for *E. cuniculi* (Ec ▼), *E. hellem* human (Eh-human ■), and *E. hellem* bird (Eh-bird ●). Chicken cells were inoculated with 1.5×10^6 spores/well and maintained at 40 °C for a period of 16 days. Lower case letters a and b represent significant differences between isolates ($P \leq 0.05$).

Isolate Growth Comparisons between Experimental Models

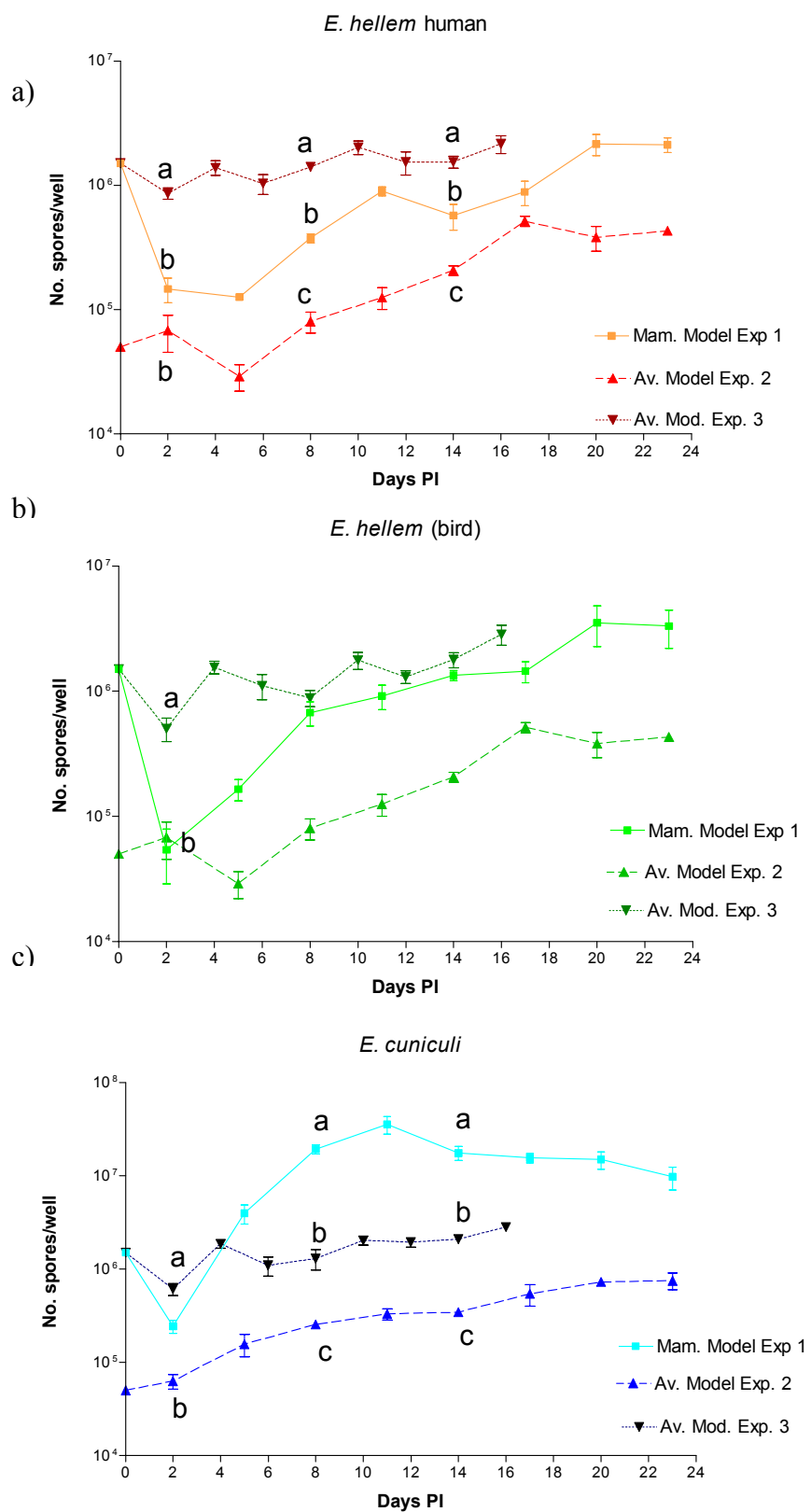
The growth curves of each isolate for each experiment were graphed separately. Additional statistical analyses were performed for each isolate comparing the average number of spores produced for each experiment at days 2, 8, and 14 PI. Days 2, 8, and 14 PI were chosen because harvest days coincided for each experiment at these days.

At day 2 PI, the results obtained for the *E. hellem* human isolate in the mammalian model Experiment 1 and avian model Experiment 2 were significantly different ($P= 0.1373$). At days 8 and 14 PI, significant differences were observed between all experiments ($P<0.005$, Appendix III). The avian model Experiment 3 had higher numbers of spores, but a typical growth curve was not observed. The total spore numbers were lower under avian model Experiment 2 compared to the mammalian model, however, a growth curve comparable to that under the mammalian model was observed.

At day 2 PI the growth of the *E. hellem* bird isolate under avian model Experiment 3 was significantly different than mammalian model Experiment 1 ($P<0.0001$) and avian model Experiment 2 ($P<0.0001$). Higher total numbers of spores were calculated under Experiment 3, but a growth curve was not observed. Significant differences were not observed at days 8 and 14 PI and the growth curves of the mammalian model and avian model Experiment 2 were comparable.

Significant differences were observed at days 2, 8, and 14 ($P\leq 0.005$) for *E. cuniculi* between mammalian and avian models. *E. cuniculi* grew significantly better under the mammalian model. Under the avian model Experiment 2 a longer lag phase is observed and the logarithmic phase of the growth curve is depressed compared to the mammalian model. A typical growth curve was not observed for Experiment 3.

Fig. 3.4. *In vitro* growth curve comparisons of *E. cuniculi* and *E. hellem* human and *E. hellem* bird isolates under different experimental models. a) *In vitro* growth curves of *E. hellem* human isolate from mammalian Experiment 1 (■) and avian model Experiments 2 (▲) and 3 (▼). b) *In vitro* growth curves of *E. hellem* bird isolate from mammalian Experiment 1 (■) and avian model Experiments 2 (▲) and 3 (▼). c) *In vitro* growth curves of *E. cuniculi* from mammalian Experiment 1 (■) and avian model Experiments 2 (▲) and 3 (▼). Days 2, 8 and 16 PI were chosen for statistical analyses because at these days spore harvest coincided.



Discussion

The purpose of the *in vitro* experiments was to create different culture conditions in order to simulate different host cell environments and to evaluate the microsporidian growth within these environments, thereby determining if the conditions in 1 model were more conducive to parasite growth. Mammalian and avian models were chosen to compare isolates of *E. hellem* and *E. cuniculi*.

E. cuniculi is a well defined mammalian microsporidia and the type species of the genus *Encephalitozoon*. Therefore, the *in vitro* growth of *E. cuniculi* under mammalian conditions compared to avian conditions would reflect the natural preferential host conditions of the microsporidian. A mammalian model known to support the *in vitro* growth of the control microsporidian, *E. cuniculi*, was chosen to evaluate the growth of *E. hellem* isolates compared to avian conditions. *E. hellem* was first described in human infections in 1991 (Didier *et al.* 1991a) and was not documented from an avian host until 1997 (Black *et al.* 1997), but subsequent cases of avian microsporidiosis have identified *E. hellem* from a variety of bird species (Carlisle *et al.* 2002). This study was designed to evaluate if *E. hellem* isolates showed preferential growth under “avian” conditions, i.e. avian cells (chicken embryonic fibroblasts) and serum (chicken) and 40 °C as opposed to “mammalian” conditions, i.e. mammalian cells (rabbit kidney) and serum (fetal bovine) and 37 °C as an indicator of natural host preference of these parasites.

The selections of *in vitro* parameters were based on previous studies and ATCC recommendations. Rabbit kidney cells, DMEM, and FBS, which were used for the mammalian model, have been used in previous studies for *in vitro* culture of both *E. cuniculi* and *E. hellem* (Didier *et al.* 1991a; Didier *et al.* 1991b; Bacchi *et al.* 2002). Although a variety of cell lines have been used for the propagation of *Encephalitozoon* spp., for the purposes of these experiments an adherent, non-rapidly replicating cell line was necessary due to the limited growth area of the 24 well plates used in the experiments. Since RK-13 cells in 24 well plates were successfully used in drug screening studies, this cell line was chosen for the mammalian model (Didier *et al.* 1998; Millership *et al.* 2001; Bacchi *et al.* 2002). The ATCC recommended incubation temperature for the RK-13 cell line at 37 °C, which is the temperature at which previous *E. hellem* and *E. cuniculi* isolates have been cultured (Visvesvara, 2002). Therefore, 37 °C was the incubation temperature selected for the mammalian model.

The chicken embryonic cell line chosen for Experiments 2 and 3 was the only adherent avian cell line available through ATCC and was therefore selected for the avian model experiments. The ATCC recommended incubation temperature for the chicken cell line is 39 °C, however, the average avian body temperature is 41 °C and, therefore, 40 °C was selected as a compromise between the 2 temperatures. Although fetal bovine serum was a recommended media supplement for both cell lines, chicken serum was used for the avian model in keeping with the avian model.

A variety of media have been used for the cultivation of *Encephalitozoon* (Visvesvara, 2002). DMEM was the recommended media by ATCC for the chicken

cell line and since RK-13 cells have been shown to be successfully maintained in different types of media, this medium was selected for both mammalian and avian models (Visvesvara, 2002).

The inocula chosen for Experiment 1, 1.5×10^6 spores/well, and Experiment 2, 5×10^4 spores/well, were based on parasite to host cell ratios. Previous *in vitro* experiments evaluating the efficacy of different antimicrosporidial compounds inoculated cell layers with either a 3:1 or 4:1 parasite to host cell ratio (Didier *et al.* 1998; Bacchi *et al.* 2002; Sobottka *et al.* 2002). Thus, for mammalian model Experiment 1, an approximate 4:1 parasite to host cell ratio was used. For avian model Experiment 2, 1×10^4 chicken cells were plated per well because of the rapid growth of the cell line. Since the cells were inoculated with the microsporidia before reaching confluency, fewer spores were inoculated per well, 5×10^4 . This resulted in a calculated 5:1 parasite to host cell ratio, however, due to the rapid growth of the cells, the actual ratio likely approached 4:1.

For avian model Experiment 3, a fixed number of spores, 1.5×10^6 spores/well, was used rather than a parasite to host cell ratio because of the low spore counts that resulted during Experiment 2. It was anticipated that this would allow a better direct comparison of growth under mammalian versus avian conditions. However, the high inoculum, approximately 150:1 spore to host cell ratio, resulted in less than favorable conditions for parasite growth and typical growth curves were not observed. In addition, a minimal increase in spore numbers was observed over the 16 day

experiment. Therefore, the results of avian model Experiment 3 are not comparable with avian model Experiment 2 or the mammalian model.

E. cuniculi grew better under the mammalian model compared to both isolates of *E. hellem*. *E. cuniculi* underwent a shorter lag phase and rapidly entered logarithmic growth (day 2) which lasted until day 11. On the other hand, the *E. hellem* isolates had a longer lag phase and the logarithmic phases of these isolates were not as pronounced as that of *E. cuniculi*. *E. cuniculi* produced a maximum number of spores at day 11 PI, which was much earlier compared to *E. hellem* isolates, which produced a maximum number of spores at day 20 PI.

In Experiment 2, where an approximate 4:1 parasite to host cell ratio for the inoculum was used, the *E. hellem* bird isolate had the highest growth curve. Although the *E. cuniculi* isolate had higher spore numbers than those of the *E. hellem* human isolate, further evaluation of the logarithmic phases of these isolates show that the *E. hellem* human isolate produced a greater number of spores compared to *E. cuniculi* during this phase. In addition, the slope of the growth curve of human *E. hellem* was comparable to that of bird *E. hellem*. Between days 5 and 17 PI, the *E. hellem* bird isolate showed a 15.5 fold increase in the number of spores produced and the *E. hellem* isolate had a 16.5 fold increase, while *E. cuniculi* only had a 4.5 fold increase.

The lower number of spores seen for the *E. hellem* human isolate under Experiment 2 was also noted in the RK seeded 75 cm² flasks used to propagate the inoculum for the experiment. This observation led to the speculation that undetected

low level contamination was present, and fresh stocks of cryopreserved spores were used to propagate all three isolates before proceeding to avian model Experiment 3.

The results indicate that *E. hellem* isolates can infect and replicate under mammalian and avian conditions. Although the results do not provide enough evidence to support preferential growth of these parasites under the avian model, the results do support the potential zoonoses of *E. hellem*. In addition, *E. cuniculi*, a well known mammalian parasite showed preferential growth under the mammalian model. However, it remains to be determined what variable or variables, i.e. incubation temperature, host cell type, or serum, under the avian model had the greatest influence on the growth of *E. cuniculi*.

Temperature, host cell type, and serum types are 3 factors affecting host parasite interactions that have been previously investigated (Taylor and Baker, 1987). For microsporidia, temperature influences both the infectivity of spores (Shadduck & Polley, 1978; Olson, 1981; Trammer *et al.* 1999), and the morphological characteristics of spores (Trammer *et al.* 1999). In a study by Trammer *et al.* (1999) the effect of temperature on the *in vitro* cultivation of *Nosema algerae*, a microsporidian parasite of mosquitoes, was observed. Parasites were grown in human muscle fibroblasts at 31 °C and 38 °C. At these 2 temperatures there were differences in the time until infected cells were detected, as well as in the number of parasites produced. Infected cells were seen after 48 hours PI when cultured at 31 °C and not until after 72 hours PI when cultivated at 38 °C. Additionally, differences in the arrangement of the polar filament within spores were also noted (Trammer *et al.*

1999). It is possible that a similar effect may have occurred in the current study so that under the mammalian model at 37 °C the *E. hellem* isolates produced a maximum spore value later than under the avian model at 40 °C. Conversely, the higher temperature of the avian model may have negatively influenced the growth of *E. cuniculi*, resulting in a logarithmic curve that was not as steep as that in the mammalian model.

Host cell types directly impact *in vitro* cultivation of parasites. For the *in vitro* cultivation of *Trypanosoma brucei*, for example, a slowly growing feeder cell line is preferred to a rapidly growing cell line. Rapidly growing cells deplete the medium of nutrients and metabolites accumulate faster, producing less favorable conditions for parasite growth (Taylor & Baker, 1987). Therefore, the rapidly replicating cells of the chicken cell line may have provided less favorable growth conditions for the isolates under the avian model. During Experiment 2, it was observed that the medium became increasingly yellow and appeared acidic by 48 hours after old medium had been removed and replaced with fresh medium. After 72 hours the medium was bright yellow suggesting a very acidic pH. The dramatic change in pH was most likely due to the increase of metabolites produced by the extremely rapid growth of the chicken cells; therefore, the interval between spore harvests was reduced from 3 days to 2 days in avian model Experiment 3. However, whether this change in protocol was helpful remains undetermined because a typical growth curve was not produced. This is likely attributable to the elevated spore to host ratio used.

In addition, the growth of *E. cuniculi* in different cell lines has been evaluated and has shown *E. cuniculi* isolates from a rabbit, mouse and hamster produced a much greater number of spores in rabbit choroid plexus cells and canine embryo cells as compared to RK-13 cells and primary hamster glial and kidney cells (Montrey, Shadduck & Pakes, 1973). A separate study by Shadduck and Polley (1978) showed that higher passaged cells were more easily infected with *E. cuniculi*. Based on those results the differences in parasite growth reported by Montrey *et al.* (1973) are most likely due to other characteristics rather than the passage level of the cells since some of the primary cell cultures produced higher number of spores than different cell lines at a higher passage. The RK-13 cell line and the chicken cell line used in the current experiments were both approximately at the same passage level (passage 168), therefore, it is more likely that other characteristics of the cells, such as replication rates, directly impacted the growth curves of the isolates used in this study.

Serum is an important supplement for the growth of the host cell as well as the growth of the parasite. FBS has been shown to support a wide range of cell lines and has been successfully used in the cultivation of different parasites from different host species (Taylor & Baker, 1987). FBS is the one of the most predominant sera used for many *in vitro* systems. FBS is versatile in that it can support a wide range of cell types and commercially available FBS is reliable in consistency (Mather & Roberts, 1998). Avian parasites of the genera *Cryptosporidium* and *Eimeria* have also been cultured in medium supplemented with FBS (Augustine, 2001; Arrowood, 2002). Therefore, a critical experiment to evaluate the avian and mammalian models would

be to use FBS in both models as the serum supplement in order to evaluate the influence of FBS on parasite growth.

In order to effectively evaluate the factors influencing the differences in growth of the bird and human *E. hellem* isolates, further experiments are needed. Future studies should include a “human” model. Ideally mammalian, human and avian cell lines that can grow equally well at 37 °C and 41 °C need to be used. This would aid in eliminating effects on microsporidian growth caused by differences in host cells. This would also allow for further evaluation of the effects of serum and temperature on the microsporidium. All of the host cell lines should be fully adapted to the same basal medium to eliminate any variation due to the medium components. The effect of serum types could further be evaluated by conducting a set of experiments using different serum types in the same medium and host cells. Or, alternatively, a serum could be selected that reliably supports the growth of the cells equally as well for each model. Also, based on the differences observed between avian model Experiments 2 and 3, a spore to host cell ratio must be maintained between experiments and the duration of the experiments should be at least 24 days.

The results of these experiments do not conclusively define preferential growth conditions of the *E. hellem* isolates; however, the results do indicate that *E. hellem* isolates can in fact infect and replicate in mammalian cells at 37 °C as well as avian cells at 40 °C, thereby indicating the probable zoonotic potential. The results of these experiments combined with previous molecular genotyping of human and bird *E. hellem* isolates and avian experimental infections show that *E. hellem* is a parasite of

zoonotic potential and the risks of owning pet birds should be explained to immunocompromised pet owners.

CHAPTER I

SUMMARY

The *E. hellem* (bird and human) aminopeptidase sequences presented here clearly show that these isolates are nearly identical, providing further molecular evidence of the high identity (>99 %) shared between these isolates. Furthermore, the *E. hellem* and *E. cuniculi* aminopeptidase sequences obtained share nearly 70% identity indicating the enzyme is conserved between species and may be a valid target for drug therapy. No substitutions were observed in regions critical to enzyme activity. The aminopeptidases presented here appear to be homologous with the *E. cuniculi* glutamyl aminopeptidase (GenBank accession no. NP_597136).

It is likely that the *Encephalitozoon* aminopeptidases characterized here are susceptible to aminopeptidase inhibitors as are other members of the M1 family and, therefore, are a potential drug target. However further characterization of the aminopeptidase is needed. The sequence data obtained in this study could be used to express a recombinant protein allowing for *in vitro* screening of different pharmaceuticals. Comparison of the recombinant protein to native protein would allow for insight into post translational modifications of the native enzyme. In addition portions of the recombinant protein may also be used to create antibodies which can later be tagged with a fluorescent label and used to localize the aminopeptidase *in vitro*. Molecular sequences have provided evidence of drug susceptibility in other organisms in which resistance to certain drugs has been correlated to mutations in amino acid sequences (De Stasio & Dahlberg, 1990; Kwa,

Veenstra & Roos, 1994; Kwa *et al.* 1995). Therefore, it would be meaningful to sequence this aminopeptidase gene from other microsporidia that infect humans, such as *E. intestinalis*, *V. corneae*, and *E. bienersi*, to predict aminopeptidase susceptibility. It would also be advantageous to investigate the role of this enzyme in the parasite life cycle and/or host cell and parasite interactions.

The results of the *in vitro* experiments do not conclusively show preferential growth of *E. hellem* under the avian model, however, the results do show that *E. hellem* can infect and replicate equally well under both avian and mammalian conditions. Although further experiments are needed to demonstrate the preferred host conditions of these parasites, the results of the *in vitro* experiments combined with the molecular data provide further evidence of the possible zoonoses of these parasites.

Although bird to human transmission has not been reported, *E. hellem* infections have been documented in humans that owned pet birds. Furthermore, parasite shedding has been documented in asymptomatic pet bird species (Barton & Snowden, 2003). Therefore, immunocompromised individuals should be aware of the risk that pet birds could be carriers of *E. hellem*.

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APPENDIX I

Average number of spores per well for *E. hellem* bird and human, and *E. cuniculi* under mammalian and avian models. The time points at which spores were harvested including the day PI which corresponded are listed.

Mammalian model Experiment 1								
Time Point	1	2	3	4	5	6		8
Days PI	2	5	8	11	14	17	20	23
Eh human	1.5x10 ⁵	1.3X10 ⁵	3.8X10 ⁵	9X10 ⁵	5.7X10 ⁵	8.8X10 ⁵	2.1X10 ⁶	2.1X10 ⁶
Eh bird	5.4x10 ⁴	1.7X10 ⁵	6.7X10 ⁵	9.2X10 ⁵	1.3X10 ⁶	1.4X10 ⁶	3.5X10 ⁶	3.3x10 ⁶
Ec	2.4x10 ⁵	4.0X10 ⁶	1.9X10 ⁷	3.5X10 ⁷	1.8X10 ⁷	1.55X10 ⁷	1.5X10 ⁷	9.7x10 ⁶

Avian Model Experiment 2								
Time Point	1	2	3	4	5	6		8
Days PI	2	5	8	11	14	17	20	23
Eh human	6.7x10 ⁴	2.9X10 ⁴	8X10 ⁴	1.2X10 ⁵	2X10 ⁵	5.1X10 ⁵	3.8X10 ⁵	4.3X10 ⁵
Eh bird	4.3x10 ⁴	1.1X10 ⁵	4.5X10 ⁵	8.5X10 ⁵	1.4X10 ⁶	1.8X10 ⁶	3X10 ⁶	1.6X10 ⁶
Ec	6.2x10 ⁴	1.6X10 ⁵	2.5X10 ⁵	3.3X10 ⁵	3.4X10 ⁵	5.4X10 ⁵	7.3X10 ⁵	7.5X10 ⁵

Avian Model Experiment 3								
Time Point	1	2	3	4	5	6		8
Days PI	2	4	6	8	10	12	14	16
Eh human	8.5x10 ⁵	1.4X10 ⁶	1X10 ⁶	1.4X10 ⁶	2X10 ⁶	1.5X10 ⁶	1.5X10 ⁶	2.2X10 ⁶
Eh bird	5.0x10 ⁵	1.6X10 ⁶	1.1X10 ⁶	8.8X10 ⁵	1.8X10 ⁶	1.3X10 ⁶	1.8X10 ⁶	2.9X10 ⁶
Ec	6.1x10 ⁵	1.9X10 ⁶	1.1X10 ⁶	1.3X10 ⁶	2X10 ⁶	2X10 ⁶	2.1X10 ⁶	2.8X10 ⁶

APPENDIX II

P-value results of statistical analyses comparing the growth of *E. hellem* human (EH), *E. hellem* bird (Mu5), and *E. cuniculi* (Ec), within experimental models.

Mammalian Model Experiment # 1				Avian Model Experiment # 2				Avian Model Experiment # 3			
Isolate	Isolate	Time Point	p- value	Isolate	Isolate	Time Point	p- value	Isolate	Isolate	Time Point	p- value
Ec	Eh	1	0.5105	Ec	Eh	1	1	Ec	Eh	1	0.1588
Ec	Mu5	1	0.0019	Ec	Mu5	1	0.1811	Ec	Mu5	1	0.295
Eh	Mu5	1	0.0087	Eh	Mu5	1	0.1811	Eh	Mu5	1	0.0214
Ec	Eh	2	<.0001	Ec	Eh	2	<.0001	Ec	Eh	2	0.1021
Ec	Mu5	2	<.0001	Ec	Mu5	2	0.1558	Ec	Mu5	2	0.2908
Eh	Mu5	2	0.5619	Eh	Mu5	2	<.0001	Eh	Mu5	2	0.5277
Ec	Eh	3	<.0001	Ec	Eh	3	<.0001	Ec	Eh	3	0.983
Ec	Mu5	3	<.0001	Ec	Mu5	3	0.0411	Ec	Mu5	3	0.9842
Eh	Mu5	3	0.034	Eh	Mu5	3	<.0001	Eh	Mu5	3	0.9672
Ec	Eh	4	<.0001	Ec	Eh	4	0.0004	Ec	Eh	4	0.3971
Ec	Mu5	4	<.0001	Ec	Mu5	4	0.0007	Ec	Mu5	4	0.4026
Eh	Mu5	4	0.7226	Eh	Mu5	4	<.0001	Eh	Mu5	4	0.1036
Ec	Eh	5	<.0001	Ec	Eh	5	0.0554	Ec	Eh	5	0.9406
Ec	Mu5	5	<.0001	Ec	Mu5	5	<.0001	Ec	Mu5	5	0.3712
Eh	Mu5	5	0.0027	Eh	Mu5	5	<.0001	Eh	Mu5	5	0.4108
Ec	Eh	6	<.0001	Ec	Eh	6	0.9912	Ec	Eh	6	0.2195
Ec	Mu5	6	<.0001	Ec	Mu5	6	<.0001	Ec	Mu5	6	0.3717
Eh	Mu5	6	0.1567	Eh	Mu5	6	<.0001	Eh	Mu5	6	0.7236
Ec	Eh	7	<.0001	Ec	Eh	7	0.0097	Ec	Eh	7	0.1063
Ec	Mu5	7	0.009	Ec	Mu5	7	<.0001	Ec	Mu5	7	0.2763
Eh	Mu5	7	0.2678	Eh	Mu5	7	<.0001	Eh	Mu5	7	0.5408
Ec	Eh	8	0.0091	Ec	Eh	8	0.0562	Ec	Eh	8	0.3673
Ec	Mu5	8	0.0288	Ec	Mu5	8	0.0044	Ec	Mu5	8	0.8012
Eh	Mu5	8	0.5362	Eh	Mu5	8	<.0001	Eh	Mu5	8	0.4963

APPENDIX III

Results of statistical analyses comparing the growth of *E. hellem* human (EH), *E. hellem* bird (Mu5), and *E. cuniculi* (Ec) between experimental models at days 2, 8, and 14 PI.

Day PI	Isolate	Exp.	Avg.# Spores	Exp.	Avg.# Spores	P-value
2	Eh	M1	1.5 x 10 ⁵	A2	6.7 x 10 ⁴	0.1373
2	Eh	M1	1.5 x 10 ⁵	A3	8.5 x 10 ⁵	<.0001*
2	Eh	A2	6.7 x 10 ⁴	A3	8.5 x 10 ⁵	<.0001*
8	Eh	M1	3.8 x 10 ⁵	A2	8.0 x 10 ⁴	<.0001*
8	Eh	M1	3.8 x 10 ⁵	A3	1.4 x 10 ⁶	<.0001*
8	Eh	A2	8.0 x 10 ⁴	A3	1.4 x 10 ⁶	<.0001*
14	Eh	M1	5.7 x 10 ⁵	A2	2 x 10 ⁵	0.0027*
14	Eh	M1	5.7 x 10 ⁵	A3	1.5 x 10 ⁶	<.0001*
14	Eh	A2	2 x 10 ⁵	A3	1.5 x 10 ⁶	<.0001*

Day PI	Isolate	Exp.	Avg.# Spores	Exp.	Avg.# Spores	P-value
2	Mu5	M1	5.4 x 10 ⁴	A2	4.3 x 10 ⁴	0.4702
2	Mu5	M1	5.4 x 10 ⁴	A3	5.0 x 10 ⁵	<.0001*
2	Mu5	A2	4.3 x 10 ⁴	A3	5.0 x 10 ⁵	<.0001*
8	Mu5	M1	6.7 x 10 ⁵	A2	4.5 x 10 ⁵	0.308
8	Mu5	M1	6.7 x 10 ⁵	A3	8.8 x 10 ⁵	0.2627
8	Mu5	A2	4.5 x 10 ⁵	A3	8.8 x 10 ⁵	0.0574
14	Mu5	M1	1.3 x 10 ⁶	A2	1.2 x 10 ⁶	0.9379
14	Mu5	M1	1.3 x 10 ⁶	A3	1.8 x 10 ⁶	0.0879
14	Mu5	A2	1.2 x 10 ⁶	A3	1.8 x 10 ⁶	0.1763

Day PI	Isolate	Exp.	Avg.# Spores	Exp.	Avg.# Spores	P-value
2	Ec	M1	2.4 x 10 ⁶	A2	6.2 x 10 ⁴	0.0065
2	Ec	M1	2.4 x 10 ⁶	A3	6.1 x 10 ⁵	0.0171
2	Ec	A2	6.2 x 10 ⁴	A3	6.1 x 10 ⁵	<.0001*
8	Ec	M1	1.9 x 10 ⁷	A2	2.5 x 10 ⁵	<.0001*
8	Ec	M1	1.9 x 10 ⁷	A3	1.3 x 10 ⁶	<.0001*
8	Ec	A2	2.5 x 10 ⁵	A3	1.3 x 10 ⁶	<.0001*
14	Ec	M1	1.8 x 10 ⁷	A2	3.4 x 10 ⁵	<.0001*
14	Ec	M1	1.8 x 10 ⁷	A3	2.1 x 10 ⁶	<.0001*
14	Ec	A2	3.4 x 10 ⁵	A3	2.1 x 10 ⁶	<.0001*

*p value significant

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